Vol. 35 No. 2 2022

Print ISSN: 0971-8184
Online ISSN: 0976-1926

Indian Journal of Plant Genetic Resources

An International Journal for Conservation and Use of Plant Diversity

ISPGR 1987

Indian Society of Plant Genetic Resources
New Delhi, India

INDIAN SOCIETY OF PLANT GENETIC RESOURCES

(Registration No. S/18336)

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- To disseminate knowledge relating to various aspects on plant genetic resources.
- To provide a forum for organizing symposia/conferences with a view to develop close relationship among the scientists engaged and interested in plant genetic resources activities.

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ISSN: 0971-8184
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INDIAN SOCIETY OF PLANT GENETIC RESOURCES
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INDIAN SOCIETY OF PLANT GENETIC RESOURCES

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Indian J. Plant Genet. Resour. 35(2): 159–168 (2022)
DOI 10.5958/0976-1926.2022.00016.X

REVIEW ARTICLE

ITPGRFA: an Appraisal as a Prelude to the Ninth Session of the Governing Body 2022, New Delhi

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(Received: 06 June, 2022; Revised: 29 July, 2022; Accepted: 30 July, 2022)

The International Treaty on Plant Genetic Resources for Food and Agriculture is a comprehensive legally binding agreement adopted during the 31st session of FAO in November 2001 at Rome. The present article is a collation of information related to key area of work of the Treaty, Committees and Working Groups of the Treaty, agenda and action taken by intergovernmental technical committees/expert groups and current scenario and prospects of the Treaty. The Governing Body (GB) is an apex body comprising of all contracting parties. The GB is responsible for policy guidance and all the decisions related to objectives and working of the Treaty. During GB meetings intersessional work of the past two years is reviewed. To date, eight sessions of GB have been convened and important decisions of the meetings are summarized here. The Ninth Session of the GB is scheduled to be held in New Delhi, India in 2022, the provisional Agenda for the Ninth Session is also elaborated in this appraisal.

Key Words: ITPGRFA, Governing Body, MLS, Intergovernmental technical committees, Expert groups

Introduction

The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) is a comprehensive legally binding agreement adopted during the 31st session of FAO in November 2001 at Rome. The Treaty came into force on 29 June 2004. Currently, the Treaty has 148 Contracting Parties including European Union (Nnadozie, 2021).

The Treaty is historic, and for the first time, the enormous contribution made by the farmers to the development and conservation of crop diversity was recognised in the Treaty. The objectives of the Treaty are:

- Conservation of plant genetic resources for food and agriculture (PGRFA)
- Sustainable use of PGRFA
- The sharing of the benefits arising from the use of PGRFA in a fair and equitable way

The text of the Treaty is organised in various Articles in ten Parts. Overall objectives and directions of the Treaty are contained in Article 1 (Moore and Tymowaski, 2005); Article 2 provides the use of terms and definitions. Article 3 is related to the scope of the treaty. General provisions contained in Part II (Article 4-8), relating to conservation and sustainable utilization of plant genetic resources for food and agriculture. Farmer's Rights are (FRs) contained in Part III (Article 9), the Multilateral System (MLS) of Access and Benefit-Sharing (MLS) contained in part IV (Article 10-13), financial provisions contained in Part V (Article 18) and supporting components contained in part VI (Article 19-35) of the Treaty (Fig. 1). The key area of work of the Treaty are represented by six different themes viz. benefit-sharing fund, global information system, sustainable use, farmer's rights, MLS and compliance. Article 4-8 of the Treaty indicates policies and measures for the national government about the conservation and sustainable use of PGRFA. Sustainable utilization is not defined in the Treaty, however, it implies making use of crop diversity to meet the food security needs of present generations, without compromising its availability, as the basis of food security for future generations (FAO, 2013). General provisions of the Treaty apply to all PGRFA, not just Annex I crops (Annex 1 crops are available for exchange under a MLS). Article 9 of the Treaty provides internationally agreed common ground for FRs, and provides measures for contracting parties to take at the national level for the protection and promotion of FRs. The Treaty provides protection of traditional knowledge, rights to participate in sharing of benefits arising out of the use of PGRFA and the right to participate in decision making related to PGRFA (FAO,

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2013). The Treaty promotes *in-situ* conservation through on-farm management of crops and *ex situ* conservation by establishing a network of gene banks. *In-situ* farm management allows crops to continuously evolve and adapt to the changing environmental conditions. *Ex-situ* conservation facilitates enhanced access and utilization, in addition to serving as a safety backup. The Treaty established a unique mechanism of the MLS as contained in Article 10-13 of the treaty. It covers 64 crops and forages listed in Annex I of the Treaty (Article 11). MLS provides farmers and breeders access to the available plant genetic resources and facilitates research and breeding for new varities required to feed the galloping population (Article 12). The contracting parties have agreed on the creation of a benefit-sharing fund arising out of the use of PGRFA under Article 13. The benefit-sharing fund supports on-farm management projects in developing countries. The Treaty has given due importance to the Global Plan of Action for conservation and sustainable use of plant genetic resources for food and agriculture (Article 14) and contracting parties should develop a coherent framework for technology transfer and exchange of information. The contracting parties recognized the importance of the *ex-situ* collections held by Consultative Groups on International Agriculture Research (CGIAR) and crops held in gene banks should be available as per the provisions MLS (Article 15). The Treaty indicated the need to encourage contracting parties to participate in international networks (Article 16) and develop a global information system to facilitate the exchange of PGRFA between contracting parties (Article 17). The Governing Body (GB), composed of all the contracting parties of the ITPGRFA, was established under Article 19. The GB is responsible for policy guidance and all the decisions of the Treaty (Fig. 1).

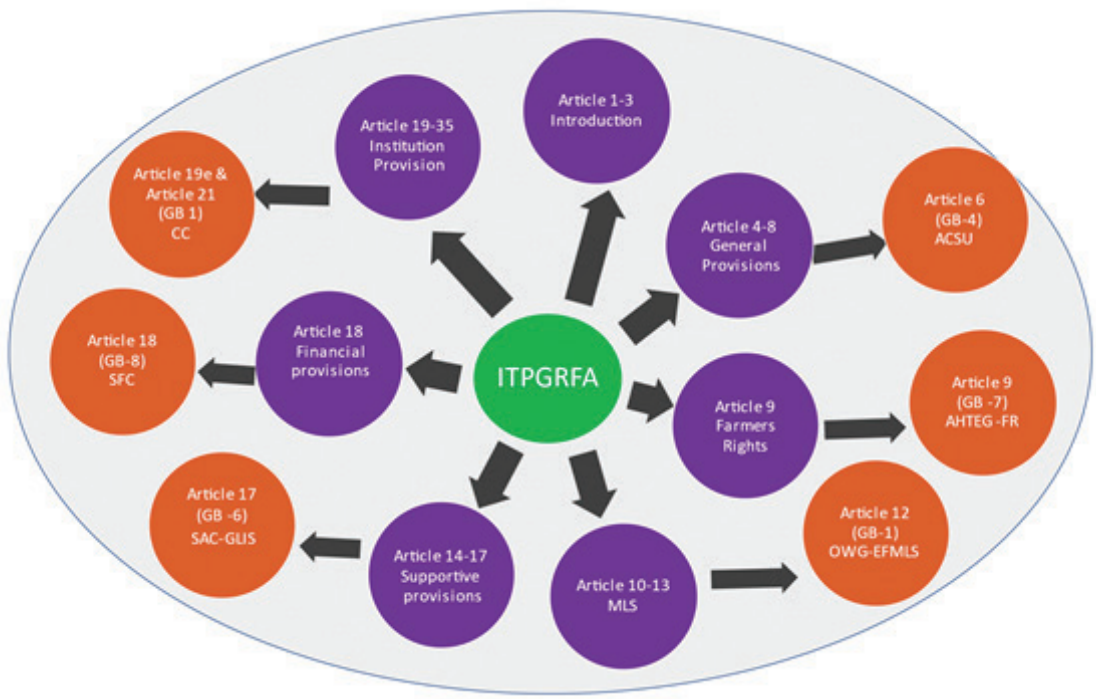

Fig. 1. Provisions and committees of ITPGRFA

GB: the Governing Body
ITPGRFA: International Treaty on Plant Genetic Resources for Food and Agriculture
ACSU: Ad Hoc technical committee on sustainable use of plant genetic resources for food and agriculture
AHTEG-FR: Ad Hoc technical expert group on farmers' rights
OWG-EFMLS: Ad Hoc open-ended working group to enhance the functioning of the multilateral system
SAC-GLIS: Scientific advisory committee on global information system
SFC: Ad Hoc Committee on funding strategy and resource mobilization
CC: Ad Hoc working group on procedures and operational mechanisms to promote compliance and address the issue of non-compliance (compliance committee).

Governing Body Sessions

The GB, convenes a meeting in alternate years to discuss various policies and plans of common interest and decisions are taken by adopting resolutions. These decisions are adopted by consensus (and not by voting), that means all contracting parties must agree to the resolution being adapted. During GB meetings work of the past two years is also reviewed; however, the consensus is always required for incorporating amendments in the Treaty (Article 23) and Annexes (Article 24). Annexes are related to the list of crops covered under the MLS and Arbitration and Conciliation. To date, eight sessions of GB have been convened and important decisions of the meetings are summarized in Table 1.

Committees and Working Groups of the Treaty

The GB has established intergovernmental technical committees/expert groups to advise the secretary on these matters (Fig. 1). These committees work between different sessions of the GB to deal with the technical and operational matters and report to the GB.

Ad Hoc technical committee on sustainable use of Plant Genetic Resources for food and agriculture (ACSU)

The GB established ACSU by Resolution 7/2011 and reconvened by subsequently with renewed terms of reference (ITPGRFA, 2022). The ACSU committee was formed to report to the secretary on the following aspects-

- Review the compilation and summaries provided by the secretary on the conservation and sustainable use of PGRFA.
- Based on this review, identify examples and opportunities to support and assist Contracting Parties and stakeholders in promoting, enhancing and

Table 1. Sessions and important decisions of the GB of ITPGRFA

GB Sessions	Important decisions/resolutions
GB Session 1 June, 12-16 2006 Madrid, Spain	A total of three resolutions were adopted 1/2006 on funding strategy 2/2006 on the SMTA 3/2006 on the compliance
GB Session 2 29 Oct.- 02 Nov 2007 Rome, Italy	A total of three resolutions were adopted 1/2007 on compliance 2/2007 on Farmer's Rights 3/2007 programme of work and budget 2008-09
GB Session 3 Tunis, Tunisia	Of the total 8 resolutions adopted, important resolutions were- 4/2009 The MLS of access and benefit-sharing 5/2009 Procedures for third party beneficiary 6/2009 Implementation of Article 9 on farmer's rights 7/2009 Cooperation with the Commission on Genetic Resources for Food and Agriculture
GB Session 4 March 14-18, 2011 Bali, Indonesia	Of the total 9 resolutions adopted, important resolutions were- **01/2011 Financial rules of the GB** **02/2011 Procedures and operational mechanisms to promote compliance and address issues of non-compliance** **04/2011 Implementation of the MLS** **05/2011 Operation of the Third Party Beneficiary** **7/2011 Implementation of Article 6, Sustainable use of plant genetic resources**
GB Session 5 Sept. 24-28, 2013 Muscat, Oman	Several resolutions and reports were discussed and the following important resolutions were adopted IT/GB-5/13/07 Add. 4 **Arrangements for the Ad Hoc Open-Ended Working Group to Expand Benefit-Sharing and the Scope of the MLS** IT/GB-5/13/09 Implementation of the Article 6 Sustainable Use of Plant Genetic Resources for Food and Agriculture IT/GB-5/13/10 Implementation of Article 9, Farmers' Rights
GB Session 6 Oct. 5-9, 2015 Rome, Italy	Several resolutions and reports were discussed and the following important resolutions were adopted IT/GB-6/15/07 Adopted vision paper on the development of the Global Information System IT/GB-6/15/09 Reviews and Assessments under the MLS and of the Implementation and Operation of the SMTA was presented and discussed
GB Session 7 30 Oct -3 Nov. 2017 Kigali, Rwanda	Several resolutions and reports were discussed and the following important resolutions were adopted IT/GB-7/17/07 Report of the Ad Hoc Open-ended Working Group to Enhance the Functioning of the MLS (Proposed for extending the mandate of Ad hoc working group on MLS and draft third revised draft of SMTA was presented and discussed

(ITPGRFA, 2006; 2007; 2009; 2011; 2013; 2015; 2017; 2019)

further developing the conservation and sustainable use of PGRFA as set out in Articles 5 and 6 of the International Treaty.

- Review the information provided by the secretary on the Toolbox for Sustainable Use of PGRFA.
- Based on this review, assess the relevance and effectiveness of the Toolbox and prepare concrete recommendations on how it can be monitored, evaluated and improved to guide better Contracting Parties and stakeholders for the sustainable use of PGRFA.
- Provide advice about the possibility of a future Joint Programme on Biodiversity in Agriculture for Sustainable Use of PGRFA based on the documentation prepared by the secretariat.

The committee has convened five meetings and has reported to the GB for consideration in the GB sessions (Table 2).

Ad Hoc Open-ended Working Group to Enhance the Functioning of the MLS (OWG-EFMLS)

The most important provision of the Treaty is a MLS and it covers 64 crops (called annexe 1 crops). These crops account for 80 per cent of all human consumption from plants. Under the MLS, the exchange of germplasm is facilitated through the standard material transfer agreement (SMTA) which is a standard bilateral contract for the transfer of PGRFA (Agrawal et al, 2013). In the GB8 session, the CGIAR consortium informed that 60,000 SMTA had been signed to exchange germplasm. SMTA was adopted by the GB, through resolution 2/2006. The GB created a committee (resolution 2/2013) "OWG-EFMLS to advise secretary on issues related to –

- Increasing user-based payments and contributions to the Benefit-sharing Fund in a sustainable and predictable long-term manner.
- Enhancing the functioning of the MLS by additional measures.

The committee provided valuable technical input to the GB for improving the functioning of MLS (Table 3).

Governing Body extended the mandate of the working groups through various resolutions (resolution 1/2015 and resolution 2/2017). MLS is the largest pool of genetic resources available for exchange worldwide. It has enabled 6 million global transfers at an average

Table 2. Meetings and agenda items of ACSU

Meeting	Agenda items
The first meeting of the ACSU Rome, Italy, 8-9 Nov. 2012	• The stakeholders' consultation • Elements for the definition of the programme of work on sustainable use of PGFRA (POW-SU) • Development of a toolbox on sustainable use of PFRFA • Farmers' rights: compilation of submissions received and report of the regional workshops
The second meeting of the ACSU 2-3 March 2015, Rome, Italy	• Implementation of article 6, "programme of work on sustainable use." • National and stakeholders' experiences regarding breeding strategies and regulations concerning variety release and seed distribution • Identification of interrelations between the international Treaty, especially article 9, and relevant instruments of UPOV and WIPO
The third meeting of the ACSU 24–25 October 2016, Vienna, Austria	• Resolution 4/2015, implementation of the programme of work on sustainable use of PGRFA • Identification of interrelations between the international Treaty, especially its article 9, and relevant instruments of UPOV and WIPO
The fourth meeting of the ACSU 8 April–5 May 2019	• The electronic consultation using the d-group online platform • Outcomes and preparation for the eighth session of the GB • Report of the electronic consultation
Expert meeting on the toolbox for sustainable use of plant genetic resources for food and agriculture 19–21 July 2016, Volterra, Italy	• Toolbox functions, toolbox content, toolbox online portal. (Toolbox will provide Contracting Parties and all interested stakeholders with a comprehensive set of resources, including technical information, policy options, regulatory guidelines, training opportunities, decision tools, and other materials to enhance the effectiveness of activities promoting the sustainable use of PGRFA).
Fifth meeting of the ACSU 4 – 7 October 2021 (online)	• Toolbox on sustainable use of PGRFA • Possible joint programme on biodiversity in agriculture for sustainable use of PGRFA • Examples and opportunities to support and assist contracting parties and stakeholders in implementing articles 5 and 6 of the International Treaty • Recommendations for further steps to assist contracting parties in advancing the implementation of articles 5 and 6 of the International Treaty

Table 3. Meetings and agenda items of (OWG-EFMLS)

Meeting	Agenda items
The first meeting of OWG-EFMLS Geneva, Switzerland 14-16 May 2014	Enhancing the functioning of the MLS of access and benefit-sharing: background on the work undertaken by the ad hoc committee on the funding strategy, and its further development Timing and preparations for future meetings of the working group
The second meeting of the OWG-EFMLS Geneva, Switzerland 9-11 Dec. 2014	Enhancing the functioning of the MLS of access and benefit-sharing Development of a range of measures to increase user-based payments and contributions to the benefit-sharing fund
The third meeting of the OWG-EFMLS Brasília, Brazil 2-5 June 2015	Measures to enhance the MLS to be considered and eventually approved by the GB , at its sixth session Interim measures to maintain the operations of the benefit-sharing fund, pending final adoption of the package of measures to enhance the functioning of the MLS of access and benefit-sharing Recommendation for 6th sessions for GB
The fourth meeting of the OWG-EFMLS Rome, Italy 5-9 October 2015	Draft revised SMTA, which is contained in Appendix 1 of this Report; Commentary on Structural Elements for the Development of a Subscription Model/System, which is contained in Appendix 2 of this Report; Possible objectives and elements of a Protocol to the International Treaty, which is contained in Appendix 3 of this Report; Exploring a proposal to develop a mechanism of contributions by Contracting Parties to the Benefit-Sharing Fund, which is contained in Appendix 4 of this Report; Submissions Received by the Working Group during the biennium, in preparation for the Subscription System and the draft revisedSMTA.
The fifth meeting of the OWG-EFMLS Geneva, Switzerland 12-14 July 2016	Elaboration of a complete draft revised SMTA focusing primarily on the development of a subscription system Enhancing the functioning of the MLS: measures beyond the elaboration of the complete draft revised SMTA
The sixth meeting of the OWG-EFMLS Rome, Italy 14-17 March 2017	Revised SMTA with a focus on the subscription system Launch mechanism for an enhanced MLS Genetic information associated with material accessed from the MLS
The seventh meeting the OWG-EFMLS Rome, Italy 5-7 September 2017	Preparations for the seventh session of the GB, including discussion of the draft resolution
The eighth meeting of the OWG-EFMLS Rome, Italy 10-12 October 2018	Revision of the SMTA of the MLS of access and benefit-sharing Elaboration of criteria and options for possible adaption of the coverage of the MLS Development of a proposal for a growth plan to attain the enhanced multilateral system
The ninth meeting of the OWG-EFMLS Rome, Italy 17-21 June 2019 and 24-26 October 2019	Revision of the SMTA of the MLS of access and benefit-sharing Adoption of the report of the working group and the report to the 8th session of the GB, including elements of a draft resolution

rate of 1000 transfers a day (Nnadozie, 2021), thus highlighting its role in ensuring equitable access and in prospective sharing of benefit. The Treaty has launched a call for proposals under benefit-sharing funds since 2009 to support vulnerable farmers (ITPGRFA, 2022). Already about 1 million people from 67 developing countries have benefited by implementing 81 projects using benefit-sharing funds generated through germplasm exchange (Nnadozie, 2021).

These projects have helped to establish local community seed banks in developing countries and strengthen the collections in gene banks for global exchange.

Scientific Advisory Committee on Global Information System (SAC-GLIS)

The enhanced benefits of germplasm exchange which is envisaged under the Treaty is possible only if the information of the stored germplasm is available to the user. The Treaty facilitated the creation of GLIS having information required to utilise stored germplasm (Article 17). The GLIS is a web portal that serves as a global entry point integrating the entire exiting gene bank data related to PGRFA. Governing Body created a scientific advisory committee in 2015 (resolution 3/2015). The scope of the committee was further extended by resolutions 5/2017 and 4/2019. The committee has convened four meetings and has advised the secretary on various matters referred to the committee as below and also detailed in Table 4.

Table 4. Meetings and agenda items of SAC-GLIS

Meeting	Agenda items
The first meeting of the SAC-GLIS San Diego, USA 7-8 January 2015	• Core PGRFA data domains and information systems • The Permanent Unique Identifiers for PGRFA • The draft Vision Paper and the elements of the Vision • Advice on the development of the Programme of Work
The second meeting of the SAC-GLIS Rome, Italy 13 – 14 June 2017	• Operations and implementation of the programme of work • Review of the guidelines for digital object identifiers • Reporting on partnerships, collaborations and capacity development
The third meeting of the SAC-GLIS Rome, Italy 21 – 22 June 2018	• Experiences and Refining Guidelines in the Application and Use of Digital Object Identifiers • Development and Promotion of Standards as Outlined in Objective 3 of the Programme of Work on the Global Information System • Master plan for the GLIS Portal • Genetic Sequence Data concerning PGRFA • Development of partnerships, collaboration and capacity building
The fourth meeting of the SAC-GLIS (virtual mode) 20–21 April 2021	• Update on the implementation of the GLIS portal • Development and promotion of digital object identifiers • Access and use of PGFRA information through the global information system and other relevant developments • Reporting on partnerships and collaboration

- General recommendations on the development and deployment of the GLIS and its components as adopted by the Governing Body
- The discovery of new areas of work with potential impact on the System
- The selection of pilot activities for the Global Information System and, upon request of the Secretary, other initiatives and actions to sustain the operation of the GLIS , and the further update of the Programme of Work
- To review, as may be required, the Programme of Work on GLIS for the consideration of the Governing Body at its Ninth Session
- To continue considering scientific and technical issues of relevance to DSI/GSD, and considering national legislation, as appropriate

Ad Hoc Technical Expert Group on Farmers' Rights (AHTEG-FR)

The Treaty recognized the enormous contribution of farmers that feed the world and provisions were made in the Treaty to safeguard their interests. AHTEG-FR was created in the 7th session of Governing Body (Resolution 7/2017). The mandate of the Expert Group was safeguarding and promoting the realization of Farmers' Rights, and the committee advise the secretary on -

(i) Produce an inventory of national measures that may be adopted, best practices and lessons learned from the realization of Farmers' Rights, as set out in Article 9 of the International Treaty

(ii) Based on the inventory, develop options for encouraging, guiding, and promoting Farmers'

Table 5. Meetings and agenda items of AHTEG-FR

Meeting	Agenda items
The first meeting of AHTEG-FR, Rome, Italy, 11-14 Sept. 2018	• Inventory of national measures that may be adopted, best practices and lessons learned from the realization of farmers' rights, as set out in article 9 of the International Treaty • Options for encouraging, guiding and promoting the realization of farmers' rights as set out in article 9 of the International Treaty
The second meeting of AHTEG-FR, Rome, Italy, 20-23 May 2019	• Inventory of national measures, best practices and lessons learned from the realization of farmers' rights, as set out in article 9 of the international Treaty.
Third meeting of the AHTEG-FR, 25–28 August 2020 (Online)	• Options for encouraging, guiding and promoting the realization of farmers' rights as set out in article 9 of the international Treaty.
The fourth meeting of AHTEG-FR (Part I) (virtual mode), 4-7 May 2021	• Options for encouraging, guiding and promoting the realization of farmers' rights as set out in article 9 of the international Treaty.
The fourth meeting of AHTEF-FR (part II) (virtual mode), 23-27 August 2021	• Options for encouraging, guiding and promoting the realization of farmers' rights as set out in article 9 of the International Treaty (the options) and the information document.

Rights as set out in Article 9 of the International Treaty.

Since its inception, AHTEG-FR has convened four meetings (Table 5). The international Treaty offers a variety of capacity development material and an inventory on Farmer's Rights with national measures, best practices, lessons learnt, with a digital version recently published on the website (Nnadozie, 2021). The expert group is currently developing options to encourage, guide, and promote farmers' rights.

Ad Hoc working group on procedures and operational mechanisms to promote compliance and address the issue of non-compliance (Compliance Committee: CC)

Governing Body established Compliance Committee to promote compliance and address issues of non-compliance" in the first session of Governing Body (Resolution 3/2006). The Rules of Procedure of the Compliance Committee were adopted by the Governing Body in its 5th session through Resolution 9/2013. The committee has the following functions.

- Consider information submitted to it regarding matters relating to compliance and issues of non-compliance.
- Offer advice and facilitate assistance, as appropriate, to any Contracting Party, on matters relating to compliance to assist it in complying with its obligations under the International Treaty.
- Assist the Governing Body in its monitoring of the implementation by Contracting Parties of their obligations under the International Treaty based on reports of the Contracting Parties following Section V of the Procedures.
- Address issues of non-compliance and identify the specific circumstances of the issue referred to it, under Sections VI to VIII of the Procedures.
- Promote compliance by addressing statements and questions concerning the implementation of obligations under the International Treaty, under Section IX of the Procedures.
- Carry out any other functions as may be assigned to it by the Governing Body according to Article 21 of the International Treaty.

The committee has convened four meetings to promote compliance to the Treaty and address issues relating to non-compliance (Table 6).

Ad Hoc Committee on funding Strategy and Resource Mobilization (SFC)

The financial resources of the Treaty are managed by adopting an appropriate funding strategy. The provision of financial resources to implement activities under the Treaty is contained in part VI (Article 18) of the Treaty. The Funding Strategy prioritises the implementation of agreed plans and programmes for farmers in developing countries who conserve and sustainably utilize PGRFA. In the Eighth Session of the GB of the Treaty, a new Funding Strategy was adopted from 2020 to 2025. The aim was to ensure that sufficient financial resources are mobilized through a range of channels to implement the International Treaty in a long-term, coordinated and effective way. The target of the Funding Strategy is to generate approximately 1 billion per year (FAO, 2022). The GB in its 8th session created SFC as a standing committee (Resolution 3/2019). The committee has conducted five meetings to draft various plans to mobilize, implement and monitor resource generation, which will be discussed in the GB 9th Session (Table 7).

Table 6. Meetings and agenda items of Compliance Committee

Meeting	Agenda items
First Meeting of Compliance Committee, Rome, Italy, 20-22 April 2013	• Draft rules of procedure of the compliance committee • Standard reporting format by contracting parties
The second meeting of the Compliance Committee, Rome, Italy, 21-22 February 2017	• Synthesis of reports received from Contracting Parties on measures taken to implement the provisions of the International Treaty, • Analysed reports received from Contracting Parties on measures taken to implement the Treaty.
Third meeting of Compliance Committee, Rome, Italy, 31 January-1 February 2019	• Consideration of reports from Contracting Parties on measures taken to implement the International Treaty • The committee reviewed the Standard Reporting Format of its Report to the GB.
Fourth Meeting of Compliance Committee, 3-4 February 2021	• Review of the outcomes of the Eighth Session of the Governing Body of relevance to Compliance. • Review under the mandate of the Compliance Committee

Table 7. Meetings and agenda items of the SFC

Meeting	Agenda items
The first meeting of the SFC, 21-23 July 2020 (Virtual)	• New funding strategy was presented • Discussed priorities for resource mobilization
The second meeting of the SFC, 17-19, Nov. 2020 (Virtual)	• The first Draft of the Operational Plan 2020-2025 was discussed • Future work for 2020-21 was presented
The Third meeting of the SFC, 23-26 February 2021 (Virtual)	• The First draft of the private Sector Food Industry Engagement Strategy 2021-25 was presented • Discussed 8th funding cycle of the Global Environment Facility (GEF-8) • Draft skeleton outline of the strategy to mobilize resources from the food processing industries was discussed • Draft Monitoring, Evaluation and Learning (MEL) Framework of the Benefit-sharing Fund was discussed
The fourth meeting of the SFC, 20-22 September 2021 (Virtual)	• The second draft of the Food Industry Engagement Strategy was discussed • The third Draft of the Monitoring, Evaluation and Learning (MEL) Framework of the Benefit-sharing Fund was discussed

Current scenario

In the eighth session of the GB, the measures to enhance the coverage of MLS of Access and Benefit-sharing and revision required in SMTA presently in use were attempted. Sh Narender Singh Tomar, Minister of Agriculture and Farmers Welfare, India, highlighted the contribution of India and informed that about 10 percent of germplasm in gene bank is of Indian origin. He reiterated the importance of plant genetic resources for research and sustainable use, however, the benefit arising from the user must be shared equitability for conservation of genetic resources. He called for an operational, pragmatic, future-ready and flexible, benefit-sharing framework, considering Digital Sequence Information (DSI) and bridging the divide between north and south (FAO 2019). The Agenda for the Ninth Session provisionally includes the following items:

1. Proposal for an Amendment of the International Treaty.
2. The MLS of Access and Benefit-sharing.
   (a) Implementation and Operations of the MLS, Implementation of Article 12.3 of the International Treaty 2 IT/GB-9/22/1.
   (b) Updates on Any Informal Consultations on the Enhancement of the MLS.
3. The Funding Strategy of the International Treaty.
4. The Global Information System.
5. Conservation and Sustainable Use of PGRFA.
6. Farmers' Rights.
7. Compliance.

The present concern and bone of contention in the Treaty is revising the coverage of crops and the inclusion of DSI. Several crops such as soybeans, groundnuts, sugar cane, the wild relatives of cassava, several fruits, tomatoes etc. have not been included in the list (David, 2002). Recent technological advancements have enabled researchers mining the freely accessible sequence data. The DSI at the international level could have extensive consequences for the future of agriculture and food security (Aubry, 2019). The DSI concept is relatively new and all concerned parties agreed upon the need for a clear definition. However, a major concern is over benefit sharing. Several attempts have been made to Tract, DSI and improve transparency on DSI (Scott and Berry, 2016; WIPO, 2018). However, with present available technologies, it is difficult to establish the genetic distinctness of any given trait, variant, or metabolite in comparison to another PGRFA (Aurbry, 2019). The divide between developed and developing counties surfaced in the GB8 on these issues. Lebanon, representing the Near East countries, stressed that an enhanced MLS should prioritize a subscription system, including an increased payment rate to meet expectations of reaping benefit sharing and integrate DSI. Rwanda, representing Africa, added that expansion of MLS will depend on effective operationalization of benefit sharing. To bridge the divide between developed and developing countries is the biggest challenge in the coming session of the GB9. The world has to produce more food to feed the expanding population, using fewer resources and under more challenging climatic conditions. The Treaty will play a crucial role in increasing productivity and meeting the first two Sustainable Development Goals (SDG) of the United Nations viz. no poverty (SDG 1) and zero hunger (SDG 2).

Divergent positions on benefit-sharing hold up MLS enhancement

The benefit-sharing component of the MLS has not been considered successful. No money from users accessing PGR from the MLS was forthcoming into the system to support projects on conservation and sustainable use in developing countries. It was proposed to revise the SMTA to enhance payments into the system, particularly from commercial users. In addition, many of the crops that attract significant research and development efforts, which potentially result in commercially successful varieties, were not included in the current MLS (Annex 1). Soybean and tomato are usually mentioned as notable examples. Expanding Annex I was proposed as the solution to enhance use of PGR as well as the flow of benefits.

By June 2019, in its 9th meeting, the Ad Hoc Open-ended Working Group to Enhance the Functioning of the MLS made significant advances, including tentative agreement to expand Annex I of the Treaty (list of crops in the MLS), and tangible progress on revising the SMTA. However, two issues viz. rates for benefit-sharing payments and digital sequence information (DSI) remained outstanding. The meeting was suspended to allow for additional time to finalize negotiations. However, in the resumed 9th session in October 2019, the developed and developing countries could not achieve a compromise on benefit sharing rates and DSI issues. This led to a serious fracture in the consensus that had been previously attained and resulted in a significant number of revisions of the draft texts. As a result, the proposals of GB5 of the Treaty to increase user-based payments to the Benefit-sharing Fund and to expand the MLS got dead-locked.

During the GB8, G-77 reiterated the stand on expansion of coverage, user based payments including subscription, Art 18.4(c) and DSI that was reflected in the Working Group. The Plenary sessions witnessed clear divide between developed countries (public sharing of information including DSI, and capacity building for its use; SMTA to focus exclusively on material, not information) and developing countries (definition of genetic resources should include genetic information; DSI, alone or in combination with material, must be part of access and benefit sharing; not addressing DSI may lead to privatization through patents of farmers' material in the MLS). CGIAR Consortium noted that the Treaty provisions on benefit-sharing from commercialization could apply to both material and information arising from such material. The CGIAR Consortium celebrated the 60,000 successful SMTAs under the MLS to date, and expressed support for a subscription-based MLS that addresses DSI. Serious concerns were expressed by African Union (making PGRFA openly available as digital sequences in exchange for a paltry portion of the enormous benefits derived by seed sector) and Civil Society (Treaty's inability to address DSI is injustice to farmers and conservers).

Unfortunately, GB8 could not debate on the Working Group report and any kind of inter-sessional negotiations and deliberations were suspended. Hence, in future, this issue requires to be taken ahead through informal consultations between parties and also needs to be addressed effectively, during GB9. The GB meeting proposed in Delhi in 2022 will be a watershed moment regarding the future of ITPGRFA.

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Indian J. Plant Genet. Resour. 35(2): 169–177 (2022)
DOI 10.5958/0976-1926.2022.00017.1

RESEARCH ARTICLE

Assessment of Genetic Diversity of Small Cardamom (*Elettaria cardamomum* M.) in India

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(Received: 22 June, 2017; Revised: 04 April, 2022; Accepted: 04 April, 2022)

As part of AICRP on Spices, a total of 200 germplasm accessions of small cardamom are being maintained at Cardamom Research Station Pampadumpara as field gene bank repository. Sixty seven cardamom accessions were studied for genetic diversity by evaluating fourteen characters for three years (2006-2009) continuously. Almost all accessions showed significant variability for the biometric and biotic stress characters. The results indicated a great amount of genetic diversity in small cardamom in the evergreen tropical forest of the Western Ghats.

Key Words: Field gene bank, Small cardamom, Western Ghats, Variability

Introduction

Cardamom, *Elettaria cardamomum* Maton, popularly called as 'Queen of Spices', and the economic part of which is the dried fruit of the perennial rhizomatous herb belonging to the family zingiberaceae. It is one of the costliest and most ancient and valuable spice crops since ancient times. The crop is indigenous to south India and Sri Lanka (Purseglove 1981) but Guatemala is the largest producer and exporter of the crop. The natural habitat of the crop used to be in the evergreen rainforests of the Western Ghats of south India at altitudes between 600 and 1500 m above MSL. Cardamom is generally cross-pollinated and propagated by seedlings and suckers; occasionally, selfing also occurs. Considerable variation is encountered in seedling progenies of cardamom (Padmini *et al.,* 2000). At present, the cardamom-growing area in India is concentrated mainly in those regions that are the natural habitat of the spices: between 8°30' and 14°30'N latitude and longitude 75-70'E. The area is an elongated tract from north to south from Sirsi of Karnataka to Thirunelvelli of Tamil Nadu. East to west, it is a narrow belt of highland distributed over the Western Ghats (Madhusoodanan *et al*., 1994).

Elettaria is a small genus having only 3-4 species spread across East and Southeast Asia. Two botanical varieties were distinguished by earlier researchers, one for the wild taxon and the other for the cultivated forms (Wardini and Thomas 1999). *E. cardamomum* var. *major* Thwaites consists of wild cardamoms that are common in Sri Lanka and southern India. *E. cardamomum* var. *cardamomum* (syn var. *minor* Watt. var. *minuscula* Burkill) consists of the cultivated cardamoms, which could be classified and named as cultivar groups. Detailed documentation of cardamom genetic resources was reported by Mayne (1951), Abraham and Tulasidas (1958) and Sudharshan *et al*. (1991). It is essential that sufficient variability for economic traits should exist in the cardamom germplasm for profitable utilization in the crop breeding programmes.

The monoculture farming practices, along with environmental degradation and urban development have contributed to the loss of plant genetic resources (Van Sloten, 1990) and therefore, erosion of these resources poses a severe threat to the world's food security in long term (FAO, 1996). Hence, characterization of genetic divergence for selection of suitable and diverse genotypes should be based on sound statistical procedures, such as D'- statistics and nonhierarchical Euclidean cluster analysis (Mahalanobis 1936; Spark 1973). These procedures characterize genetic divergence using the criterion of similarity or dissimilarity based on the aggregate effect of a number of economically important characters. In view of these, cardamom genotypes were evaluated in this study to determine the magnitude of variability in the population for yield and yield components as well as the grouping pattern of genotypes in different clusters.

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Materials and Methods

The germplasm materials consisting of 67 accessions (Table 1) collected from cardamom growing areas in south India were used in this study. These accessions were evaluated at the Cardamom Research Station, Kerala Agricultural University, Pampadumpara which is located in a medium rainfall zone (1500-2500 mm per annum) at an elevation of 1100 m above MSL.

The experimental field was planted with a spacing of 2m×2m comprising 10 experimental plants per accession. The recommended crop-growing practices (Package of Practices, Kerala Agricultural University, 2006) were followed uniformly to all accessions. Data on 14 characters were recorded from 10 plants based on the IPGRI descriptor of small cardamom during 2006 to 2009 and the average were taken for statistical analysis. The mean and coefficient of variation were calculated as per the standard statistical procedures.

Results and Discussion

Significant differences among germplasm accessions were observed for all traits that indicates the presence of higher variability. Previous authors have also reported the significance difference in various characters of cardamom and other crop genotypes. Korikanthimath *et al*. (2000) reported significant difference among cardamom genotypes particularly for number of capsules per plant, weight of fresh and dry capsule and oleoresin content. Ankegowda and Krishnamurthy (2008) have also showed variability in number of tillers, number of leaves and plant height that were significantly different for six cardamom germplasm accessions under moisture stress condition.

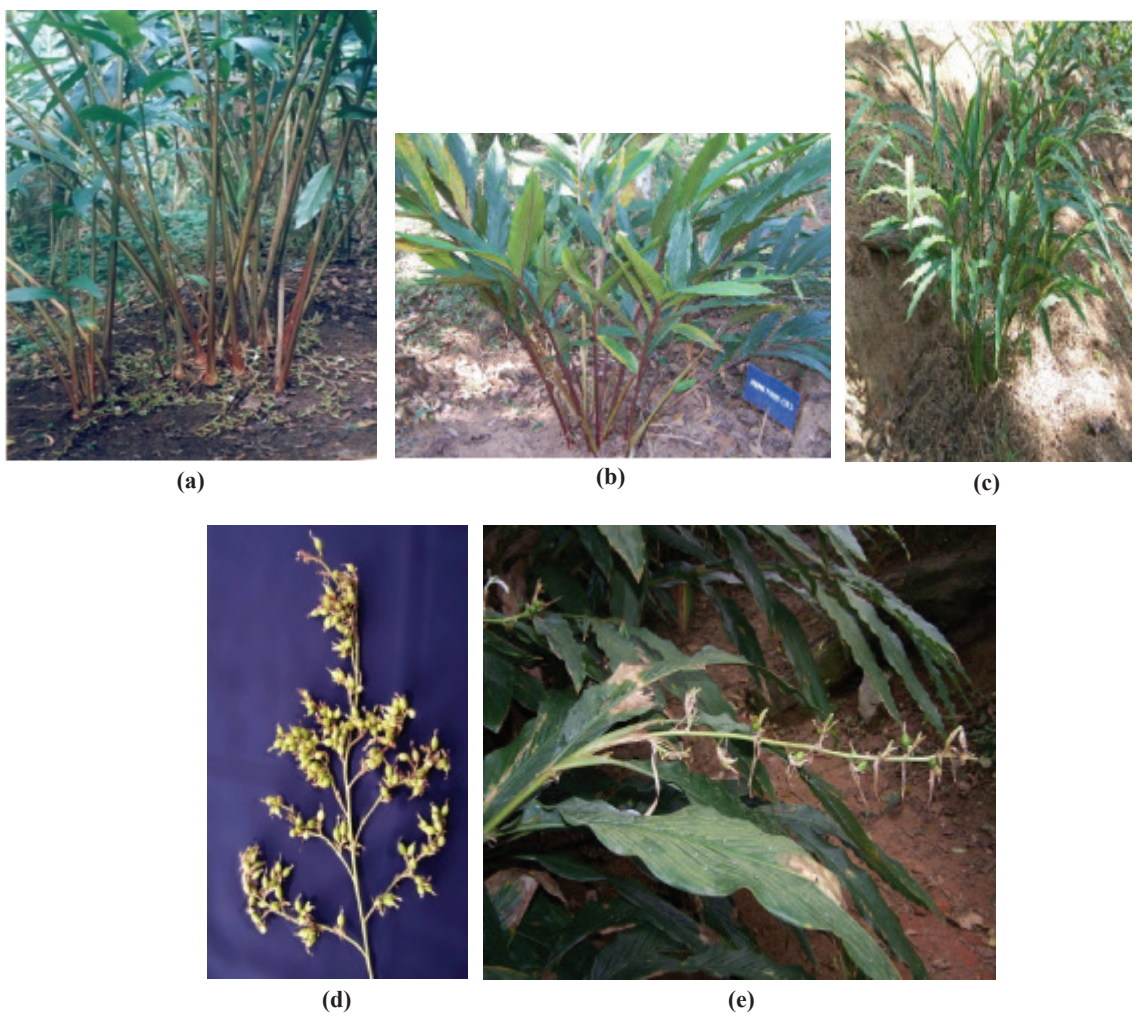

Fig. 1. Some small cardamom correlation- (a) Pink Base; (b) Mini Pink; (c) PV 8 (d) Multi branded panicle of MBP (e) Terminal panicle produced from Alfred clone

Table 1. Details of the cardamom accessions used in the genetic diversity analysis.

Sl. No.	Name of the land race/variety	Type	Source	Remarks
1	ACC 1	Vazhukka		Around 3 panicles per tiller, small globose shaped light green capsules
2	ALFRED CLONE	Malabar	From farmers' field	Presence of both terminal and basal panicle
3	BEP 1	Vazhukka	Selection from farmers' field	Close internodes between raceme
4	BEP 2	Vazhukka		Four panicles per tiller, light green colour capsules
5	CHETTI 1		Selection from farmers' field	Short stature
6	CHETTI 2			Tolerant to pest and diseases
7	CHETTI 3			
8	CLONE 37	Malabar	From IISR Regional Station, Appangala	Released as Appangala 1
9	CLONE 57			Globose light green capsule
10	COM.PAN	Malabar	Collection from Kodagu	Branched panicles
11	GREENGOLD	Vazhukka	Famers variety of Idukki	High yield with enhanced fertilizer response
12	HEMA	Vazhukka		Seventeen seeds per capsule
13	MANJURABAD	Malabar	From Shakleshpur	Three to four panicles per tiller
14	MBP	Malabar	Collection from Kodagu	Multibranched panicles
15	MCC 11		From ICRI Myladumpara	
16	MCC 40	Malabar	From ICRI Myladumpara	High yielder
17	MCC 61	Vazhukka	From ICRI, Myladumpara	Released as ICRI 2
18	MINI PINK	Malabar		Pink colouration at the base of the pseudostem
19	PINK BASE	Malabar		Pink colouration in the pseudostem
20	PPK 1	Vazhukka	Selection from farmers field	Long bold capsules
21	PPK 2	Vazhukka	Selection form green gold , Farmers variety	Extra long panicle but smaller capsules
22	PRO 17			Light green coloured rhizhome
23	PRO 107			Lower surface of leaf is glabrous
24	PS 10	Malabar	Plant selection from CRS	Elongated green colored bold capsules
25	PS 12	Vazhukka	- do -	Three panicles per tiller
26	PS 13	Malabar	- do -	Globose light green capsules
27	PS 14	Malabar	- do -	Round and light green coloured bold capsules
28	PS 16	Vazhukka	- do -	Elongated light green bold capsules
29	PS 17	Malabar	- do -	Light green coloured capsules
30	PS 18	Vazhukka	- do -	Elongated light green coloured capsules
31	PS 19	Vazhukka	- do -	Elongated green capsules
32	PS 2	Malabar	- do -	Bold elongated light green coloured capsules
33	PS 21	Vazhukka	- do -	Elongated light green capsules
34	PS 22	Vazhukka	- do -	Light green coloured bold capsules
35	PS 23	Malabar	- do -	Elongated green capsule
36	PS 24	Malabar	- do -	Green elongated bold capsules
37	PS 25	Malabar	- do -	Elongated light green capsules
38	PS 26	Malabar	- do -	Around fourteen seeds per capsule
39	PS 27	Malabar	- do -	High yielder with bold capsules
40	PS 28	Malabar	- do -	Green elongated capsules
41	PS 29	Malabar	- do -	Green elongated bold capsules
42	PS 30	Malabar	- do -	Bold elongated light green colored capsules
43	PS 31	Vazhukka	- do -	Bold elongated green colored capsules
44	PS 32	Malabar	- do -	Bold elongated green coloured capsules
45	PS 4	Malabar	- do -	Green elongated bold capsules

Sl. No.	Name of the land race/variety	Type	Source	Remarks
46	PS 5	Malabar	- do -	Green elongated bold capsules
47	PS 7	Malabar	- do -	Light green globose shaped capsules
48	PS 8	Vazhukka	- do -	Light green globose shaped capsules
49	PS 9	Vazhukka	Plant selection from CRS	Elongated green capsules
50	PV 10	Mysore	- do -	Light green coloured rhizome
51	PV 11	Vazhukka	- do -	Glabrous leaves
52	PV 12	Vazhukka	- do -	Elongated bold green coloured capsules
53	PV 2	Vazhukka	- do -	Bold elongated light green coloured capsule
54	PV 33	Malabar	CRS Pampadumpara	Light green elongated capsule
55	PV 34	Malabar	CRS Pampadumpara	Higher volatile oil content
56	PV 5	Malabar	- do -	Light green coloured globose shaped capsules
57	PV 6	Malabar	- do -	Dark green coloured capsules
58	PV 7	Malabar	- do -	Poor yielder
59	PV 8	Mysore	- do -	Narrow leaf lamina
60	PV1	Malabar	CRS Pampadumpara	Released variety with long thin capsules
61	S 1	Malabar	Seedling selection from commercial plantation of CRS	Higher yielder with long bold dark green capsules
62	Sinchona sel	Mysore		Light green coloured capsules
63	Type 1	Malabar	Seedling selection from the Malabar types	Around eighteen seeds per capsule
64	Type 103	Malabar	Seedling selection from the Malabar types	Around ten seeds per capsule
65	Type 4	Malabar	Seedling selection from CRS Pampadumpara	Light green coloured rhizome
66	Type 6	Malabar	Seedling selection from CRS Pampadumpara	Globose shaped light green coloured capsules
67	VEERAPUTHRAN	Malabar	From ICRI Myladumpara	Released variety as ICRI 1

Peculiar types in terms of physical appearance

Among all the landraces, morphologically peculiar types identified were Pink Base, Mini Pink, MBP, Alfred clone, Compound Panicle and PV8. Pink Base and mini pink were easily distinguishable from other landraces/ varieties due to their pink coloration in the pseudostem (Fig. 1). However, in the case of Mini Pink, Pink coloration is uniformly spread in the pseudostem and for Pink Base, pink colour is mainly concentrated on the base of the pseudostem. PV 8 could be distinguished by its narrow leaf lamina. All these three land races were poor yielders. MBP and compound panicle types were also categorized under land races that have multi branched panicles. Both of them were higher yielders due to their branched nature of panicles which bear more flowers. Alfred clone produces both basal and occasionally terminal panicles but found to be poor yielders when comparing it with other land races.

Variability in yield and biotic stresses

Almost all the cultivars showed significant differences in fresh capsule yield at 1% and 5% levels. Among cultivars the highest wet weight was reported by PPK 2 (3051 kg/ha) but it was on par with other cultivars like Sinchona Sel, PS 27, PS 10, MBP, BEP 1, BEP 2, CHETTI 1 and PS 28. The ultimate and penultimate lowest yield was reported by Pink Base and PV 6, respectively.

The highest dry yield recorded for PS 28 (560 kg/ha) was on par with PS 10 and PPK 2 and the lowest yield was recorded from the Pink Base. The data on the wet and dry capsule yield showed poor yielding nature of the cultivar. Weight of 100 capsules recorded for clone 57 was maximum (116 g) followed by PS 22 and PPK 1 but they were statistically at par with each other. Capsule weight was minimum for PS 7 (73 g)

Table 2. Pooled analysis (2006-2008) of yield and biotic stress characters.

Name of the cultivar	Wet weight	Dry weight	100 capsule weight	Incidence of thrips	Incidence of borer	Incidence of Azhukal
ACC 1	1373.500	297.000	97.000	21.656	0.707	1.171
ALFRED CLONE	922.500	163.000	92.500	19.358	0.977	0.707
BEP 1	2103.500	456.500	104.000	12.494	0.707	0.707
BEP 2	2034.000	445.000	94.000	25.411	1.171	1.225
CHETTI 1	2074.500	319.000	75.000	27.271	0.977	0.707
CHETTI 2	1137.000	209.000	105.500	20.889	0.977	0.707
CHETTI 3	930.500	172.000	99.000	25.741	1.171	1.171
CLONE 37	421.500	87.500	89.000	16.430	0.707	0.707
CLONE 57	1772.500	90.500	**116.000**	18.787	0.707	1.171
COM.PAN	1610.166	367.500	104.000	13.554	1.331	0.977
GREENGOLD	1865.000	399.000	103.500	25.463	**1.728**	0.707
HEMA	210.300	46.500	76.500	15.105	0.977	0.707
MANJURABAD	1016.500	187.500	98.000	26.176	1.559	1.225
MBP	2001.000	405.500	99.000	20.257	1.225	0.707
MCC 11	1060.500	200.500	100.500	25.411	1.595	0.707
MCC 40	910.500	178.500	94.500	17.171	0.977	0.707
MCC 61	1170.000	185.000	101.500	21.837	1.171	1.171
MINI PINK	289.500	63.000	88.000	23.033	0.977	**1.331**
PINK BASE	**160.500**	**34.625**	95.000	17.948	1.470	0.707
PPK 1	1623.000	334.500	**115.000**	21.898	1.693	0.707
PPK 2	**3051.000**	509.000	81.000	22.396	0.707	1.171
PR 17	456.000	103.000	83.500	16.833	1.171	0.707
PRO 107	259.500	59.000	92.500	22.455	0.707	0.707
PS 10	2635.500	**558.000**	107.000	18.824	0.707	0.707
PS 12	2166.500	454.500	104.000	20.584	0.977	1.171
PS 13	1891.500	411.000	102.500	14.535	1.693	0.707
PS 14	1566.750	319.000	92.500	18.265	1.171	0.707
PS 16	2580.500	194.000	86.000	14.049	0.707	0.707
PS 17	412.150	79.000	85.000	**7.759**	0.977	0.707
PS 18	1383.500	203.500	95.500	19.166	0.707	0.707
PS 19	1022.500	207.500	94.000	33.825	1.331	0.707
PS 2	1571.000	322.350	94.000	19.533	0.707	0.707
PS 21	1208.500	233.500	111.000	12.746	1.581	0.977
PS 22	1655.500	273.500	**115.500**	19.166	1.581	**1.331**
PS 23	1337.333	376.000	104.000	11.477	1.470	0.707
PS 24	887.500	163.000	99.000	25.161	1.331	0.707
PS 25	995.000	184.000	104.000	**45.407**	0.707	0.707
PS 26	1515.000	319.000	101.000	28.308	0.707	0.977
PS 27	2478.500	459.500	98.500	15.956	0.707	0.707
PS 28	**3018.000**	**560.000**	100.000	28.956	0.977	0.707
PS 29	1016.000	201.500	102.500	10.611	1.225	0.977
PS 30	493.500	89.500	87.500	13.481	0.977	0.707
PS 31	630.000	139.000	82.500	15.240	1.331	0.977
PS 32	1175.500	234.000	86.500	18.741	1.171	0.707
PS 4	1321.000	263.500	88.500	10.765	1.559	0.707
PS 5	1488.000	331.500	93.500	15.889	0.977	0.707
PS 7	692.000	113.500	**73.000**	11.021	0.707	0.707

Name of the cultivar	Wet weight	Dry weight	100 capsule weight	Incidence of thrips	Incidence of borer	Incidence of Azhukal
PS 8	676.000	107.000	91.500	14.707	1.693	0.977
PS 9	1011.500	224.500	91.500	20.495	1.171	1.171
PV 10	1643.000	367.500	91.000	14.764	0.977	0.707
PV 11	620.500	131.500	89.000	17.948	0.707	0.707
PV 12	1321.500	266.500	103.500	15.846	**1.728**	0.707
PV 2	918.500	204.000	92.000	25.489	0.707	0.707
PV 33	718.000	139.500	91.000	26.720	0.707	0.707
PV 34	476.000	90.000	96.500	19.680	0.707	0.707
PV 5	407.250	92.500	84.000	16.739	1.559	0.977
PV 6	**161.500**	35.000	83.500	22.384	0.707	0.977
PV 7	249.500	42.166	**74.000**	18.421	1.225	0.707
PV 8	422.500	139.500	**75.000**	17.777	0.707	0.707
PV1	905.000	186.000	90.500	29.374	1.225	0.977
S 1	1291.000	225.900	100.000	18.880	1.171	0.977
Sinchona sel	2516.500	440.500	106.500	23.292	0.707	0.977
Type 1	855.000	155.500	106.000	20.889	1.171	0.707
Type 103	1027.500	203.000	103.000	17.441	0.977	0.707
Type 4	609.000	136.000	100.500	16.078	1.171	0.977
Type 6	256.000	49.500	112.000	18.824	0.707	0.707
VEERAPUTHRAN	655.500	149.000	98.500	14.049	0.707	0.707
CV	50.203	68.197	31.431	32.641	28.704	24.1
CD (1%)	1266.21	350.814	65.540	13.494	0.647	0.425
CD (5 %)	963.42	266.92	49.864	10.267	0.492	0.323

which was at par with PV 7 and PV 8. PPK 2 and PS 28 can be recommended for breeding programmes on the account of their higher yield potential.

None of the cultivars was free from pests and disease incidence however, some of the cultivars showed significant difference in pest and disease infestation both at 1% and 5% level. This indicated that cultivars were susceptible to various biotic stresses at varying levels. Incidence of thrips infestation ranged from 7.759 % in PS 17 to 45.407% in PS 25. In the case of capsule borer incidence, infestation ranged between 0.707 % and 1.728%. Green Gold and PV 12 showed the highest borer incidence. However Green gold was the most popular variety cultivated in Idukki district of Kerala. Varied level of incidence of Azhukal disease among cultivars/landraces was shown, but the highest level of the disease incidence was shown by PS 22 and Mini pink.

Variability in Yield Attributing Characters

The accession Veeraputhran registered highest plant height. Among these characters, the highest variability was noticed for number of capsules/plant (64.40%) followed by panicle length and number of panicles/plant. According to Padmini *et al*. (2000) the highest variability was noticed in *Malabar* accessions where in the number of panicles per plant varied greatly. MBP recorded the highest number of capsules/plant (3139.2) which was mainly attributed to its branched nature of panicles. The number of panicles per plant was maximum in Veeraputhran (46.66). Moderate variability was reported on the number of internodes per panicle, number of panicles per tiller and number of tillers per plant. Type 103 registered the highest number of internodes per panicle (38) and highest panicle length. The least variability was shown for plant height and number of seeds per capsule. Backiyarani *et al*. (2000) have confirmed least coefficient of variance for the number of seeds per capsule.

Cluster Analysis

Agglomerative hierarchical clustering analysis has been done using the mean values of fourteen characters including the yield attributing and biotic stresses. The accessions studied were grouped into fourteen clusters which show the magnitude of variability. This may have arisen through decades of domestication events occurring

Table 3. Pooled data (2006-2008) of yield attributing characters.

Accession	Plant height(cm)	No. of tillers/ plant	No. of panicles/ plant	No. of capsules/ plant	Panicle length (cm)	No.of panicles / tiller)	Number of internodes/ panicle	No. of seeds /capsule
ACC 1	266.66	28.66	12	466.3	42	3	22	16.1
Alfred clone	152.66	44.66	3.33	501.3	39	3	22	16.7
BEP 1	295.33	27.66	18.33	1355.5	24	4	19	16.5
BEP 2	225	40.33	13	815.6	45	3	21	14.7
CHETTI 1	282	34.33	28.33	1041.3	25	2	19	12.3
CHETTI 2	265.66	19.66	15	1114.7				13.3
CHETTI 3	268.33	32.33	13	954.6	24	3	24	15.8
CLONE 37	256	35	18	1186.6	50	4	25	14.9
CLONE 57	228	41.66	18.33	1067.2	43	2	22	13.1
COM. PAN	329.33	45.33	23	1502.2	85	3	34	14.7
GREEN GOLD	255.33	26.33	8.33	1451.6	19	2	16	15.3
HEMA	269.66	34	16.6	889	71	3	29	16.8
MANJURABAD	220.33	48.66	8.6	924.3	25	3	17	17.1
MBP	328.66	32.33	31	**3139.2**	69	2	34	11.8
MCC 11	266.2	26	21.66	2010.6	34	2	25	14.4
MCC 40	292	29	10	1041.2	19	1	16	11.7
MCC 61	241.33	28.33	5	156.3	12	1	7	13.1
MINI PINK	306.66	21	14.66		43	3	21	11.7
PINK BASE	245	24	9.33	203.3	55	3	1	14
PPK 1	213.66	51	24	739	30	3	14	12.7
PPK 2	261	32.33	31	1050.6		3	18	14.4
PRO107	291	32.66	23.66	1111.6	29	2	19	16.2
PRO17	260	23	23	450.6	14	2	11	16.6
PS 10	301	36	16.33	1637.6	39	3	20	11.5
PS 12	325	22.66	31.33	1895.9	49	3	20	20.3
PS 13	214	26.66	8.66	991.6	28	2	17	14.3
PS 14	210.66	38.33	7.33	790.9	42	4	22	12.8
PS 16	218.33	24	12	1158.6	39	3	20	15.4
PS 17	215	40.33	14.33	298	38	2	21	14.9
PS 18	330	24.66	40.66	783.2	47	2	12	12.9
PS 19	251.66	5.33	27.33		39	4	28	14.1
PS 2	270.66	23.33	3.66	1141.6	43	1	17	13.3
PS 21	274.66	18	5.66	673.3	21	2	20	19.9
PS 22	278.66	18.66	23.66	1207.8	63	1	17	15.4
PS 23	239.66	35	21.66	920	42	2	19	13.5
PS 24	295.33	30	33	1817.9	30	3	20	14.4
PS 25	264.33	35.66	20.66	409.3	63	2	33	13.7
PS 26	229	32	23	1356.9	18			14.3
PS 27	284.33	27.33	17	1433.5	23	3	19	11.3
PS 28	224	37.33	15.33	576.6	17	2	16	13.7
PS 29	265	32	28	1687.6	34	3	21	12.4
PS 30	252.33	31	20.33	779.9	34	2	25	10.4
PS 31	312.66	26	30.33	2050.5	60	2	24	17.3
PS 32	244	24	11	110	30	2	18	12.5
PS 4	290	24	23.33	1852.6	85	4	19	14.8
PS 5	318.33	24.6	15	1188.3	43	3	37	16.4

Accession	Plant height(cm)	No. of tillers/ plant	No. of panicles/ plant	No. of capsules/ plant	Panicle length (cm)	No.of panicles / tiller)	Number of internodes/ panicle	No. of seeds /capsule
PS 7	288.3	31.66	36	1621.9	26	2	16	14.1
PS 8	296.66	31.33	16.66	430.5	30	2	13	10.6
PS 9	358	29	33.66	2674.4	45	2	21	15.6
PV 33	332.33	15.33	17.66	813.6	42	3	24	15.1
PV 34	269	23.33	11	529	20	2	16	12.8
PV1	156.33	31.33	4	474.5	15	2	8	9.6
PV10	234	**46.33**	14.66	132.9	26	2	16	9.4
PV11	207.66	37.66	27	273	19	1	11	12.5
PV12	216.66	62	8.6	261.9	33	2	19	13.5
PV2	220	35	20.3	558.2	40	4	11	12.1
PV5	277.33	43	17	589.2	68	2	23	14.1
PV6	242.33	37	24.33	719.9	50	2	21	15.3
PV7	194	31.33	8		14	2	10	10
PV8	215	44	12.66	281	10	2	9	8.1
S -1	340.66	33.66	37.33	2464	60	4	28	17.3
Sinchona sel.	254.33	20	20	861.6	29	1	14	18.5
TYPE 1	294	53.66	29	602.3	30	2	16	18.5
Type 103	271.66	25	19.66	700.2	**90**	4	**38**	10.6
TYPE 4	282	33.66	16.3	267	24	2	15	11.9
TYPE 6	211	27	5	256.3	44	3	24	10.9
Veeraputhran	**356**	38.33	**46.66**	1936.3	48	3	22	14.8
Mean	263.3679	31.71269	18.86	1005	38.26	2.476	19.630	14.010
SD	43.77519	9.566578	9.5737	647.9	18.21	0.831	6.913	2.46620
CV	16.62131	30.16641	50.737	64.40	47.6	33.55	35.218	17.602

Table 4. Cluster details of landraces/cultivars.

Cluster Number	Accessions included	Number of accessions	Central accession
1	ACC 1, PRO 17	2	ACC 1
2	Alfred clone, BEP 2, Chetti 1, Chetti 3, Hema, MCC 40, PPK 1, PRO 107, PS 14, PS 19, PS 23, PS 30, PS 5, PV 1, PV 6, Sinchona Sel.	16	Sinchona Sel.
3	BEP 1, Chetti 2, Clone 37, Clone 57, Compound panicle, Green gold, Manjuarabad, MBP, MCC 11, Mini pink, PPK 2, PS 10, PS 12, PS 13, PS 16, PS 2, PS 22, PS 24, PS 26, PS 27, PS 29, PS 31, PS 4, PS 7,PS 9, S1, Veeraputhran	27	PS 7
4	MCC 61	1	MCC 61
5	Pink base	1	Pink base
6	PS 17, PS 25, PS 8	3	PS 25
7	PS 18, PS 21, PS 28, PV 33, PV 34, PV 2, PV 5, PV 7, Type 1	9	Type 1
8	PS 32	1	PS 32
9	PV 10	1	PV 10
10	PV 11, PV 8	2	PV 11
11	PV 12	1	PV 12
12	Type 103	1	Type 103
13	Type 4	1	Type 4
14	Type 6	1	Type 6

from different parts of Cardamom Hill Reserve. However there was no definite clustering based on the source from which these accessions were collected. It may be due to the use of seedlings as planting material in the initial years of commercial cultivation of cardamom. No definite clustering was observed for peculiar genotypes. This revealed low correlation between the biometrical and morphoqualitative characters. Accessions from the same cultivar group *(Malabar/Vazhukka/Mysore)* were scattered in different clusters. This indicated the possibilities of a common ancestral type and close relationship of the genotypes of these three groups and also that the geographical origin was not the single factor for genetic divergence in cardamom (Prasath and Venugopal, 2004).

Cluster 3 was the largest one with 27 accessions (Table 4) and showed high level of genetic interrelationship among them. Therefore hybridization between these accessions may not lead to the production of progenies with high hybrid vigour due to their genetic similarity. The cluster consisted of Green Gold, PPK 2, Clone 37, Veeraputhran, PS 27, Mini Pink etc. The central accession in Cluster 3 was PS 7. Pink Base, MCC 61, PS 32, PV 10, PV 12, Type 103, Type 4 and Type 6 have formed separate clusters individually. This indicated that the variability from other cultivars with respect to its yield and yield attributing characters were different. Maximum inter-cluster distance existed between cluster 8 and 3 (1489) followed by cluster 9 and 3 (1466).

Conclusion

The present study focused on intra specific variation in cardamom with respect to yield and yield attributing characters. The accessions collected in South India and conserved at the Cardamom Research Station are an important genetic reservoir of variability. The collected germplasm accessions are now conserved in ex situ as field gene banks. Thus, there is an excellent opportunity to bring about improvement through direct selection and hybridization which can involve crossing of genotypes from different clusters. The cluster analysis showed that there was no definite clustering based on cardamom types or centre of diversification. The information gathered in this study could be used as a guide for further breeding programmes in cardamom.

Acknowledgement

The authors sincerely acknowledge the financial support rendered by the Indian Council of Agricultural Research, New Delhi, for conducting these studies under the AICRP on Spices.

Conflict of interest

The authors declare no conflict of interest.

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Indian J. Plant Genet. Resour. 35(2): 178–184 (2022)
DOI 10.5958/0976-1926.2022.00018.3

RESEARCH ARTICLE

Genetic Divergence Assessment through K-Means Clustering and Principal Component Analysis for Seed Yield, Zinc, Iron and Protein Content in *Vigna unguiculata* L. Walp.

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(Received: 08 June, 2021; Revised: 22 October, 2021; Accepted: 26 October, 2021)

Cowpea [*Vigna unculata* (L.) Walp.] with chromosome number 2n=24 is an one of the important crops grown in arid and semi-arid regions of the world. Cowpea is a source of high quality protein consumed in many parts of the world. Malnutrition is a global problems needs to be addressed locally. Identification of micronutrient dense genotypes is the major objective of cowpea Biofortification programme. In the present study, 169 cowpea genotypes collected from different sources were analysed for genetic divergence through K-means clustering followed by Principal component analysis (PCA). Considering mean values, genotypes were classified into eight clusters following K-means clustering algorithm. Cluster VII has highest number of genotypes (56 genotypes) followed by cluster III (43 genotypes). Clusters VI and VIII were solitary with single genotype. Results revealed that crosses could be made between genotypes of cluster V & VIII, cluster III & VIII and cluster VII & VIII. According to PCA, Five principal components (PC) with Eigen values greater than one contributed 70.5% of the total variability. Amongst, PC_1 accounted highest proportion of total variance (31.3 %), remaining PCs *viz*., PC_2, PC_3, PC_4 and PC_5 revealed 14.6%, 10.1%, 7.7% and 6.8 % respectively, revealing pod yield^{-1}, seed yield^{-1}, pod length and seeds pod^{-1} were major contributors to the total genetic variability and divergence.

Key Words: Cowpea, Genetic divergence, K-means clustering, Principal Component Analysis (PCA)

Introduction

Cowpea (*Vigna unguiculata* L. Walp) with chromosome number 2n=24 belonging to Leguminaceae family is widely grown in many parts of the world and has multifarious uses as pulse, vegetable, green manure and fodder. It is a one of major sources of calories, protein and minerals in developing countries (Ira *et. al*, 2020). Cowpea dry seeds (per 100g) contains carbohydrates 59.54g, protein 24g, iron 9.95mg, zinc 6.11 mg and low saturated fat 0.5g (https://www.nutritionvalue.org/Cowpeas%2C_raw%2C_mature_seeds%2C_catjang_nutritional_value.html?size=100+g). The focus of green revolution during 1960's was to improve yield levels of major food crops namely, wheat, rice and maize. The dwarf varieties, which occupied the most of cultivation area, helped to fight hunger. Monoculture of these crops resulted in unforeseen rise in malnutrition (Howarth and Ross, 2010). Most essential micronutrients vital to growth and development include iron, zinc whose deficiency can cause serious health problems like stunted growth and anaemia. Though several options are available for development of biofortified cowpea, development of biofortified cowpea via breeding approaches is a economical, viable, environmentally friendly and sustainable option. Identification of micronutrient dense genotypes is an essentially important step for development of biofortified cowpea varieties. This is only possible though a careful selection parents which are genetically divergent. The genetic improvement of cowpea begins with the selection of parents and formation of the base population generating segregating populations in which superior lines are selected, making knowledge on the dissimilarity between the parents particularly important. Several multivariate techniques are helpful to predict genetic divergence such as clustering method and/or principal component analysis. Cluster analysis, a technique of multivariate analysis, groups the plants based on their traits, so that the internal homogeneity and the external heterogeneity of the ensuing groups should be high. Similarly principal component analysis technique

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Table 1. List of cowpea genotypes used in the study and their source

Sl. No.	Genotype	Source	Sl. No.	Genotype	Source	S;. No.	Genotype	Source
1	PV-3-1	NBPGR, Jodhpur	48	EC-472250	NBPGR, Jodhpur	95	ArkaGarima	IIHR, Bengaluru
2	MFC-09-15	VC Farm, Mandya	49	PKB-4-1	GKVK, Bengaluru	96	IIHR-144	IIHR, Bengaluru
3	EC-458480	IIPR,Kanpur	50	MFC-09-10	VC Farm, Mandya	97	NBC-19	NBPGR, Delhi
4	IC-402180	IIPR, Lucknow	51	NBC-8	NBPGR, Delhi	98	EC-458473	IIPR,Kanpur
5	EC-458473	NBPGR, Jodhpur	52	EC-458402	IIPR,Kanpur	99	C-720	IIPR,Kanpur
6	IC-249588	IIPR,Kanpur	53	202804(83)	IIPR,Kanpur	100	NBC-47	NBPGR, Delhi
7	EC-402159	IIPR,Kanpur	54	CB-10	NBPGR, Jodhpur	101	NBC-43	NBPGR, Delhi
8	IC-2591054	IIPR,Kanpur	55	MS-4	IIPR,Kanpur	102	IC-202781	IIPR,Kanpur
9	TOME-774	NBPGR, Jodhpur	56	PGCP-6	Pant Nagar, UP	103	C-720	NBPGR, Jodhpur
10	PV-1-3	NBPGR, Jodhpur	57	MFC-09-17	VC Farm, Mandya	104	CHILORE-11	Shimoga local
11	AV-2-2	GKVK, Bengaluru	58	PGCP-3	Pant Nagar, UP	105	NBC-30	NBPGR, Delhi
12	MFC-09-19	VC Farm, Mandya	59	PGCP-12	Pant Nagar, UP	106	IC-402125	IIPR,Kanpur
13	MFC-09-16	VC Farm, Mandya	60	Pant lob-3	Pant Nagar, UP	107	EC-170584	NBPGR, Jodhpur
14	EC-458505	IIPR,Kanpur	61	Pant lob-2	Pant Nagar, UP	108	NBC-39	NBPGR, Delhi
15	IC-249593	NBPGR, Jodhpur	62	PGCP-5	Pant Nagar, UP	109	EC-402104	IIPR,Kanpur
16	MFC-09-20	VC Farm, Mandya	63	Pantlob-1	Pant Nagar, UP	110	IC-402180	IIPR,Kanpur
17	C-457	IIPR,Kanpur	64	PGCP-27	Pant Nagar, UP	111	EC-472252	IIPR,Kanpur
18	IC-202711(58)	IIPR,Kanpur	65	AV-5	GKVK, Bengaluru	112	IC-402090	NBPGR, Jodhpur
19	IT38956-1	NBPGR, Jodhpur	66	PKB-1	GKVK, Bengaluru	113	C-24-1	IIPR,Kanpur
20	MFC-09-8	VC Farm, Mandya	67	PKB-2	GKVK, Bengaluru	114	MFC-09-05	VC Farm, Mandya
21	IC-21483	IIPR,Kanpur	68	PV-3	NBPGR, Jodhpur	115	IT-97154299	IIPR,Kanpur
22	AV-5-1	GKVK, Bengaluru	69	PKB-4-2	GKVK, Bengaluru	116	IC-1071	IIPR,Kanpur
23	NBC-44	NBPGR, Delhi	70	EC-170584	IIPR,Kanpur	117	V-16	NBPGR, Jodhpur
24	NBC-38	NBPGR, Delhi	71	IIHR-140	IIHR, Bengaluru	118	IC-202867	NBPGR, Jodhpur
25	MFC-09-3	VC Farm, Mandya	72	IC-201	NBPGR, Jodhpur	119	EC-170604	NBPGR, Jodhpur
26	PV-1-4	NBPGR, Jodhpur	73	IIHR-137	IIHR, Bengaluru	120	C-33	Shimoga local
27	IC-402114	IIPR,Kanpur	74	PKB-3	GKVK, Bengaluru	121	MFC-09-02	VC Farm, Mandya
28	V-585	IIPR,Kanpur	75	MBC-25	IIPR,Kanpur	122	IC-202781	NBPGR, Jodhpur
29	IC-402101	IIPR,Kanpur	76	NBC-16	NBPGR, Delhi	123	NBC-24	NBPGR, Delhi
30	MFC-09-7	VC Farm, Mandya	77	PV-1	NBPGR, Jodhpur	124	IC-201	NBPGR, Jodhpur
31	Genotype-36	NBPGR, Jodhpur	78	IC-402106	NBPGR, Jodhpur	125	27749(25)	IIPR,Kanpur
32	PV-3-4	NBPGR, Jodhpur	79	EC-458438	IIPR,Kanpur	126	IC-402101	NBPGR, Jodhpur
33	IC-4506	NBPGR, Jodhpur	80	Kashi Kanchan	NBPGR, Jodhpur	127	GC-3	IIPR,Kanpur
34	IC-402161	IIPR,Kanpur	81	AV-1	GKVK, Bengaluru	128	IC-402180	IIPR,Kanpur
35	IC-4506	IIPR,Kanpur	82	IC-1061	IIPR,Kanpur	129	NC-32	NBPGR, Jodhpur
36	C-24-1	IIPR,Kanpur	83	PKB-5	GKVK, Bengaluru	130	EC-394839	NBPGR, Jodhpur
37	ETC-27	NBPGR, Jodhpur	84	APC-1218	IIPR,Kanpur	131	MBC-25	IIPR,Kanpur
38	EC-458473	IIPR,Kanpur	85	IC-58905	IIPR,Kanpur	132	MFC-09-20	VC Farm, Mandya
39	EC-458489	NBPGR, Jodhpur	86	Bhagyalakshmi	IIPR,Kanpur	133	IC-49586	NBPGR, Jodhpur
40	NBC-51	NBPGR, Delhi	87	AV-6	GKVK, Bengaluru	134	EC-277	IIPR,Kanpur
41	PKB-4-3	GKVK, Bengaluru	88	NBC-21	NBPGR, Delhi	135	EC-458483	IIPR,Kanpur
42	AV-2-1	GKVK, Bengaluru	89	IC-25105	NBPGR, Jodhpur	136	IC-202781	NBPGR, Jodhpur
43	MFC-09-18	VC Farm, Mandya	90	Goa local	IIPR,Kanpur	137	EC-458440	NBPGR, Jodhpur
44	MFC-09-4	VC Farm, Mandya	91	AV-2	GKVK, Bengaluru	138	V-585	IIPR,Kanpur
45	PV-3-2	NBPGR, Jodhpur	92	PKB-4	GKVK, Bengaluru	139	IIHR-133	IIHR, Bengaluru
46	PV-1-2	NBPGR, Jodhpur	93	NBC-32	NBPGR, Delhi	140	EC-458490	IIPR,Kanpur
47	NBC-27	NBPGR, Delhi	94	NBC-30	NBPGR, Delhi	141	NBC-42	NBPGR, Delhi
142	NBC-14	NBPGR, Delhi	152	IC-206240	NBPGR, Jodhpur	162	IC-202781	IIPR,Kanpur

Sl. No.	Genotype	Source	Sl. No.	Genotype	Source	S;. No.	Genotype	Source
143	EC-458453	IIPR,Kanpur	153	EC-170604	IIPR,Kanpur	163	CP-98	IIPR,Kanpur
144	MFC-09-01	VC Farm, Mandya	154	IC-202777	NBPGR, Jodhpur	164	CPD-15	NBPGR, Jodhpur
145	IC-257428	NBPGR, Jodhpur	155	EC-458490	IIPR,Kanpur	165	NBC-12	NBPGR, Delhi
146	EC-458511	NBPGR, Jodhpur	156	C-33	IIPR,Kanpur	166	IC-202825	IIPR,Kanpur
147	IC-249141	NBPGR, Jodhpur	157	IC-402135	NBPGR, Jodhpur	167	IC-402175	IIPR,Kanpur
148	IT-97K499-38	IIPR,Kanpur	158	NBC-21	NBPGR, Delhi	168	EC-458418	IIPR,Kanpur
149	PKB-6	IIPR,Kanpur	159	EC-458483	NBPGR, Jodhpur	169	IC-402159	NBPGR, Jodhpur
150	IT38956-1	IIPR,Kanpur	160	EC-458480	NBPGR, Jodhpur			
151	KM-5	IIPR,Kanpur	161	V-604-7-29-3	NBPGR, Jodhpur			

groups the sets of germplasm accessions, especially valuable for screening large number of descriptor variables. The tenet of present study was to find out the genetic diversity among collected genotypes and to identify better combinations as selection criteria for developing high yielding cowpea genotypes, which may help the plant breeders by promoting new opportunities for development of cowpea cultivars with better yield.

Material and Methods

The experimental material consisted of 169 cowpea accessions which were collected from research stations of Karnataka [MRS (Hebbal), UAHS (Shivamogga), College of Agriculture, VC Farm (Mandya) and AICRP on Arid Legumes, ZARS, UAS (Bengaluru) Table 1].

Experimental design and data analysis

The entries were planted during August 2015 at College of Sericulture Chintamani (weather data given in Supplementary Table 2) and field experiment was laid out in a 13 x 13 simple lattice design with two replications. Each replication consisted of thirteen sub-blocks with thirteen genotypes in each sub block. Entries and sub blocks were randomized. Each genotype was grown in one row of 2-meter length. A spacing of 45 cm between row and 15 cm between plants was maintained. Five randomly selected plants from each the 169 genotypes were used for recording observations on days to first flowering, days to fifty *percent* flowering, plant height, primary branches per plant, clusters per plant, pods per cluster, pods per plant, pod length, seeds per pod, pod yield per plant, 100-seed weight, days to maturity and seed yield per plant. Prior to the initiation of experiment, the surface soil (0-15 cm) sample was collected for the determination of fertility status. Analysis of the sample was carried out in Department of Soil Science and Agricultural Chemistry, UAS, GKVK, Bengaluru. The results are presented in the Supplementary table 1. Dry seeds of cowpea were used to estimate iron content, zinc content and protein content in seeds using Absorption Spectrophotometry (AAS) method and diacid mixture procedure as proposed by Lindsay and Norvell (1978). Five gram of dry cowpea seed flour sample was used analyze crude protein as described in the method no. 920.87 of the Association of Official Analytical Chemists (AOAC, 1995). The cowpea genotypes were classified following model-based k-means clustering approach as suggested by Mac queen, 1967 to unravel organization of variability using SAS 9.3 version software programme. Dendrogram based hierarchical clustering with the help of R function hclust function is constructed. Multivariate approach, D^2 statistic, commonly used for classifying genotypes based on their genetic distance has no control over number clusters to be formed sometimes leads ambiguity in interpretation of results. In case of k-mean clustering, one can customize on number of clusters to be formed thus meaning validation of results are possible. Data ordination multivariate approach, principal component analysis (PCA) computed following Rao (1964) as a linear combination of optimally weighted observed variables.

Results and Discussion

K-means clustering

K-mean clustering is a widely used one of the clustering algorithm helps grouping genotypes into different clusters with control over number of clusters to be formed. One hundred and sixty-nine cowpea genotypes were grouped into eight distinct clusters following 'k-means' clustering algorithm (Mac queen, 1967) and are represented in Table 2. Highest number of genotypes were included in cluster VII (56 genotypes) followed by cluster III (43 genotypes), cluster V (35 genotypes), cluster IV (25 genotypes), cluster I (5 genotypes), cluster II (3 genotypes). Clusters VI and

Table 2. List of genotypes under different clusters formed by k-mean clustering from 169 genotypes of cowpea

Cluster	Number of genotypes	Name of genotype
I	5	IC-4506, PV-1-2, MS-4, EC-277, PKB-6
II	3	MFC-09-15, AV-6, IC-25105
III	43	IT38956-1, IC-21483,PKB-4-3, PGCP-3, PGCP-5, Pant lob-1, AV-5, PV-3, PKB-4-2, IIHR-140, PV-1, IC-1061, PKB-5, APC-1218, Goa local, AV-2, IIHR-144, NBC-19, EC-458473, IC-402125, EC-170584, NBC-39, EC-402104, IC-402180, EC-472252, C-24-1, IC-1071, V-16, MFC-09-02, IC-202781, NBC-24, IC-201, IC-402101, NC-32, EC-394839, MBC-25, IC-49586, EC-458483, EC-458440, V-585, NBC-42, EC-458453, IC-249141
IV	25	PV-3-1, MFC-09-16, EC-458505, C-457, MFC-09-8, NBC-44, PV-1-4, IC-402114, Genotype-36, PV-3-4, IC-4506, IC-402161, ETC-27, EC-458473, EC-458489, NBC-51, AV-2-1, MFC-09-18, MFC-09-4, EC-472250, MFC-09-10, EC-170584, MFC-09-05, IC-402180, CP-98
V	35	IC-249593, MFC-09-20, IC-202711(58), NBC-38, C-24-1, NBC-8, 202804(83), PKB-3, NBC-21, C-33, MFC-09-20, IC-202781, IIHR-133, NBC-14, IC-257428, EC-458511, IT-97K499-38, IT-38956-1, KM-5, IC-206240, EC-170604, IC-202777, EC-458490, C-33, IC-402135, NBC-21, EC-458483, EC-458480, V-604-7-29-3, CPD-15, NBC-12, IC-202825, IC-402175, EC-458418, IC-402159
VI	1	PKB-4-1
VII	56	EC-458480, IC-402180, EC-458473, IC-249588, EC-402159, IC-2591054, TOME-774, PV-1-3, AV-2-2, MFC-09-19, AV-5-1, MFC-09-3, V-585, IC-402101, MFC-09-7, PV-3-2, NBC-27, EC-458402, CB-10, PGCP-6, MFC-09-17, PGCP-12, Pant lob-3, Pant lob-2, PGCP-27, PKB-1, PKB-2, IC-201, IIHR-137, MBC-25, NBC-16, IC-402106, EC-458438, Kashi Kanchan, AV-1, IC-58905, PKB-4, NBC-32, NBC-30, Arka Garima, C-720, NBC-47, NBC-43, IC-202781, C-720, CHILORE-11, NBC-30, IC-402090, IT-97154299, IC-202867, EC-170604, 27749(25), GC-3, EC-458490, MFC-09-01, IC-202781
VIII	1	Bhagyalakshmi

Table 3. Mean comparison profile of cowpea germplasm lines into different clusters by K-mean clustering method

Characters	Clusters and number of genotypes in each cluster								F Value	P Value
	C1	C2	C3	C4	C5	C6	C7	C8		
	5	3	43	25	35	1	56	1		
Days to first flowering	58.50	49.00	57.58	48.70	50.93	55.00	50.49	47.50	6.29**	0.00
Days to 50% flowering	61.70	51.83	60.69	51.68	53.90	58.00	53.44	50.50	6.33**	0.00
Plant height (cm)	108.64	75.13	128.13	89.29	81.15	74.15	79.57	112.26	23.95**	0.00
Primary branches/plant	5.46	5.43	5.33	5.43	5.19	5.13	5.11	6.88	1.09	0.37
Clusters/plant	8.45	8.84	7.75	9.71	9.72	6.75	8.12	5.63	1.70	0.11
Pods/cluster	1.56	1.44	1.47	1.61	1.57	1.38	1.49	1.19	2.73*	0.01
Pods/plant	13.29	14.29	12.56	16.64	16.92	9.00	13.29	8.13	2.57*	0.01
Pod length (cm)	16.02	17.11	16.77	16.95	16.60	20.11	16.46	15.43	0.56	0.78
Seeds/pod	13.07	13.24	13.92	13.62	13.80	15.13	13.28	14.63	0.50	0.83
Pod yield/plant (g)	20.61	13.15	17.98	26.34	26.71	11.34	19.90	12.65	3.14**	0.00
100-seed weight (g)	10.80	10.83	10.26	10.70	10.30	12.00	10.69	11.00	0.37	0.91
Days to maturity	89.60	86.83	87.92	87.00	87.04	88.00	85.96	85.00	1.07	0.38
Iron content (ppm)	11.81	7.61	9.78	14.03	14.47	4.96	10.77	7.34	3.05**	0.00
Zinc content (ppm)	384.76	616.53	122.65	218.04	57.69	231.20	133.12	848.00	382.99**	0.00
Protein content (%)	18.00	18.67	35.66	14.51	16.65	192.00	28.10	17.40	34.97**	0.00
Seed yield per plant (g)	23.81	24.35	24.58	24.01	24.10	25.54	24.34	23.40	0.97	0.45

VIII were solitary clusters. The traits mean differences between clusters were significant for most of the traits (Table 3) except for primary branches per plant, clusters per plant, pod length, seeds per pod, hundred seed weight, days to maturity and protein content. The estimates of the means of pod length, seeds per pod, hundred seed weight, zinc content and protein content were highest among the genotypes were included in cluster VI. While, days to first flowering, days to fifty *percent* flowering, clusters per plant, pods per cluster, pods per plant, pod

length, days to maturity and protein content had lowest estimates of the means for genotypes grouped under cluster VIII. Genotypes from different geographical origin distributed into all the clusters indicating that there is no relationship between geographical distribution and genotypic diversity. Though wide range of phenotypic variability observed in the study, clustering of genotypes from different sources grouped under many clusters might be due less genetic distance between them.

Inter cluster values and Inter relation of clusters

It would be desirable to attempt crosses between genotypes belonging to distant clusters for getting highly heterotic crosses (Venkatesh et al., 2017). But, heterosis cannot be exploited in a highly self-pollinated crop like cowpea (Yadav et al., 2010) on commercial scale. However, the crosses involving parents from cluster with high inter cluster distance are likely to yield desirable recombinants in the advanced generation, which could be developed as traditional homozygous varieties. In this context, inter cluster distances were worked out considering the quantitative characters and the distances varied from 51.35 (between clusters III and VII) to 791.50 (between V and VIII). The inter cluster distances was maximum between cluster V and VIII (791.15) followed by cluster III and VIII (725.94) and between cluster VII and VIII (715.79). These values suggest that the genotypes from distant clusters exhibit wide diversity (Falconer, 1981). Thus, genotypes from divergent clusters can be selected for hybridization programme for obtaining desirable recombinants.

The nearest and farthest cluster from each individual clusters are presented in Supplementary table 3. The inter-cluster distances varied from 51.35 (between cluster III and VII) to 791.15 (between clusters V and VIII). All the other inter-cluster distances were lying between these values. Cluster I consisting of 5 genotypes was nearer to clusters IV (168.63). Cluster II comprised of 3 genotypes was close to cluster VIII (234.56). It was farthest from cluster V (559.10). Cluster III, IV and V were grouped with 43, 25 and 35 genotypes respectively, which were closer to clusters VII (51.35, 86.97 and 76.81). Cluster VI had only one genotype in its group. It was nearer to clusters IV (179.93). Clusters VII comprised of 56 genotypes and was nearer to cluster III (51.35). Cluster I, III, IV, V, VI and VII are farthest from cluster VIII (463.67, 725.94, 630.65, 791.15, 642.29 and 715.79 respectively). Cluster VIII was grouped with single genotype, which was closer to the cluster II (234.56) and was farthest from cluster V (791.15).

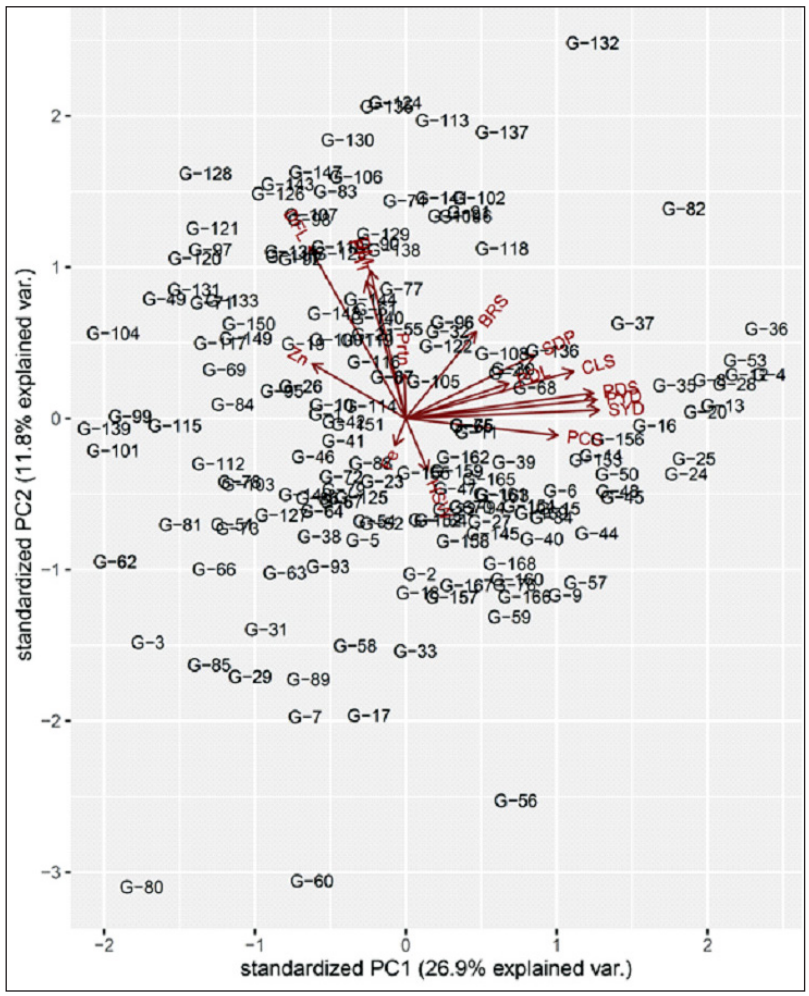

Fig. 2. Distribution of genotypes across PC1, PC2

Cluster mean analysis

Cluster mean values for all characters are presented in Table 3 indicate that clusters differ with respect to mean performance of zinc, iron, protein, yield and its attributes. This reflects the clusters formed are very distinct. The genotypes grouped under cluster I and II consisting of five and three genotypes respectively

showed high mean values for days to first flowering, days to *percent* flowering and days to maturity. Cluster VI with single genotype (PKB-4-1) represented high mean values compared to other clusters for character pod length, seeds per pod, hundred-seed weight, zinc content and protein content. Two clusters (VI and VIII) with single genotypes each were widely diverse for days to first flowering, plant height, pod length, seed yield per plant, iron content and zinc content. Genotype Bhagyalakshmi recorded lowest cluster mean for days to first flowering, days to fifty *percent* flowering, clusters per plant, pods per cluster, pods per plant, pod length, days to maturity and protein content was grouped under cluster VIII.

Highest mean value for pods per cluster was recorded for genotypes classified under cluster IV. It is worthy to note that calculating cluster means, the superiority of a particular genotype in respect of a given character get diluted by other genotype that are related and grouped in the same cluster which are interior or intermediary for that character in question. Hence, apart from selecting lines from cluster which have high cluster distance for hybridization, one can also think of selecting parents based on the extent of divergence in respect to a character of interest. This means that if breeder's intention is to improve the seed yield to plant then selection should be aimed at parents which are highly divergent for the trait of interest. The clusters VI and VIII that are having diverse mean values of most of the characters can be used as parents in hybridization programme aimed at improving the trait in consideration. These findings were in line with the reports of Dalsaniya *et al*. (2009) and Sandeep *et al*. (2014).

Principal component analysis

Principal component analysis was performed to identify the most contributing variables to the total variability observed (Fig. 2). Five principal components with Eigen values greater than one contributed 70.5 *percent* of the total variability among 169 genotypes evaluated for 16 traits including three nutritional traits. The following traits contributed positively to PC_1 that had 31.3 *percent* variation proportion to the total variability: pod yield per plant (0.408), pods per plant (0.401) and seed yield per plant (0.396). In PC_2 accounting for 14.6 *percent* of the total variability, days to first flowering (0.512), days to fifty *percent* flowering (0.511) and days to maturity (0.430) contributed positively to the total variation.

The traits pod length (0.581), seeds per pod (0.487) and zinc content (0.341) contributed positively to the total variation, which is noticed in PC_3 that accounts for 10.10 *percent* of total variation. PC_4 had 7.7 *percent* variation of the total variation where iron content (0.583) and 100-seed weight (0.452) contributed positively and negatively by protein content (-0.463) to the variation. In PC_5, protein content (0.563) and primary branches per plant (0.484) contributed positively while 100-seed weight (-0.359) contributed negatively to the variation. From the entire five principal components pod yield per plant, seed yield per plant, pod length and seeds per pod contributed significantly to the total genetic variability and divergence.

It revealed that pod yield per plant, seed yield per plant, pod length and seeds per pod contributed significantly to the total genetic variability and divergence. These results corroborate those of earlier reports by Singh *et al.* (2008), Muhammad lawan umar (2014) and Udensi and Edu (2015).

Conclusions

K-means clustering was opted to organize the genetic variability present in a set of 169 genotypes. The values ranged from 51.35 to 791.15. Grouping the genotypes into clusters using K-means clustering method resulted in the formation of 8 clusters of which cluster VII was the biggest with 56 entries followed by cluster III, V, IV and I with 43, 35, 25 and 5 genotypes respectively. The mean differences between clusters were significant for the traits days to first flowering, days to fifty *percent* flowering, plant height, pods per cluster, pods per plant, pod yield per plant, seed yield per plant, iron content and zinc contents. The inter cluster distance varied from 51.35 (between clusters III and VII) to 791.15 (between V and VIII). The estimates of the means of the traits such as pod length, seeds per pod, hundred seed weight, zinc content and protein content were highest among the genotypes included in cluster VI. Traits like days to first flowering, days to fifty *percent* flowering, clusters per plant, pods per cluster, pods per plant, pod length, days to maturity and protein content had lowest estimates of the means in cluster VIII. Thus genotypes from the clusters VI and VIII are found to be genetically divergent and contrasting which could be utilized in the hybridization programme.

Principal component analysis identified the most contributing variables to the total variability. Five principal components with Eigen values greater than one contributed 70.5 *percent* of the total variability among 169 genotypes evaluated for 16 traits including three nutritional traits. Pod yield per plant, seed yield per plant, pod length and seeds per pod contributed significantly to the total genetic variability and divergence. Thus, priority should be given these characters when selection is exercised in cowpea crop improvement programmes. Performing genetic divergence studies before starting up a new breeding programme will help to determine the amount of genetic diversity present in it, thereby reducing the cost of money and time in crop improvement programmes.

Estimates of iron, zinc and protein aided in identification of nutrient efficient genotypes in the selected population of 169 cowpea genotypes. Based on the mean composition of these nutrient traits, genotypes were classified into different categories. Twelve genotypes (PKB-4-1, MFC-09-17, PKBB-2, EC-170584, NBC-47, C-720, CHILORE-11, NBC-39, EC-402104, MFC-09-05, 27749-25 and NBC-24) identified as zinc, iron and protein rich.

Acknowledgement

MRS (Hebbal), UAHS (Shivamogga), College of Agriculture, VC Farm (Mandya) and AICRP on Arid Legumes, ZARS, UAS (Bengaluru).

*Supplementary Tables or Figures mentioned in the article are available in the online version.

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Supplementary Table 1. Physico-chemical properties of soil of the experimental site

Sl. No.	Physico-chemical properties	Results
I.	**Physical properties**	
1	Coarse sand (%)	48.15
2	Fine sand (%)	28.30
3	Silt (%)	14.15
4	Clay (%)	9.40
5	Soil textural class	Sandy clay loam
6	Field capacity (%)	16.60
7	Permanent wilting point (%)	5.80
8	Bulk density (g cc^{-1})	1.35

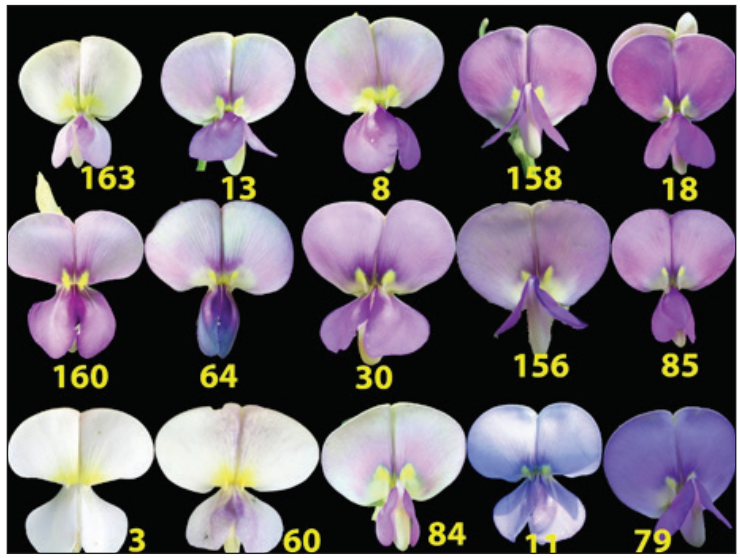
Sl. No.	Physico-chemical properties	Results
II.	**Chemical properties**	
1	pH	6.35
2	Electrical conductivity (dSm^{-1})	0.32
3	Organic carbon (%)	0.61
4	Available N (kg ha^{-1})	368
5	Available P_2O_5 (kg ha^{-1})	41.3
6	Available K_2O (kg ha^{-1})	298
7	Iron (ppm)	26.7
8	Zinc (ppm)	2.5

Supplementary Table 2. Meteorological data of Agriculture Research Station, Chintamani from January to December 2015

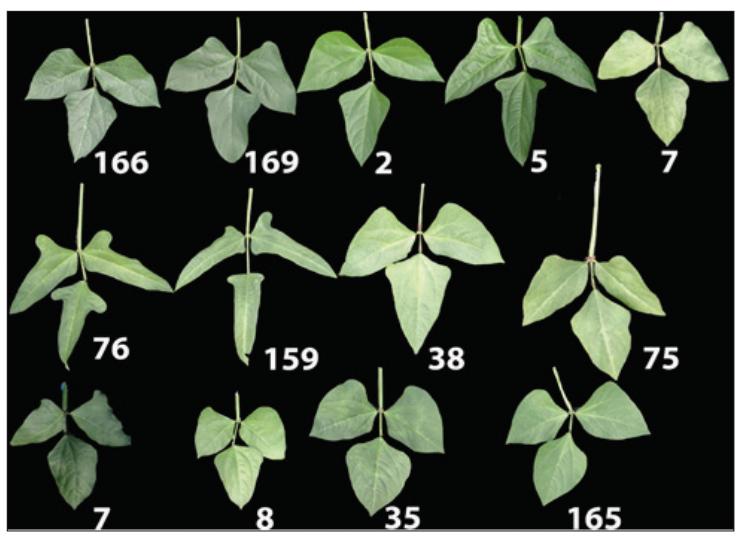
Month	Total Rain Fall (mm)	No. of rainy days	Temperature (°C)		Relative Humidity (%)		Water Evp	SS Hours
			Minimum	Maximum	Morning	Evening		
January	6.2	1	16.86	27.66	73.00	39.12	3.49	4.54
February	–	–	17.29	29.99	63.70	34.28	5.30	9.66
March	35.2	4	21.32	32.93	68.77	49.48	5.27	8.28
April	170.5	5	21.95	33.39	64.70	48.96	4.60	7.94
May	160.9	6	23.12	31.78	65.80	51.25	3.45	6.95
June	40.1	5	21.47	34.95	70.60	57.16	3.05	5.68
July	60.0	5	20.53	28.44	65.12	53.96	2.98	6.48
August	162.3	10	20.61	30.35	72.83	59.48	2.01	6.10
September	189.8	8	20.27	30.08	71.20	56.83	2.47	5.99
October	113.3	7	19.08	30.29	71.54	59.54	2.76	7.69
November	349.1	17	17.10	26.92	79.86	72.23	1.51	3.26
December	3.0	–	16.03	27.80	74.58	64.90	3.27	6.57
Total	1284.2	68						

Supplementary Table 3. Principal component analysis showing the contribution of morphological and nutritive traits to the total variation among the cowpea accessions

Communality	PC_1	PC_2	PC_3	PC_4	PC_5
Days to first flowering	-0.243	0.512	-0.062	-0.003	-0.079
Days to 50% flowering	-0.244	0.511	-0.063	-0.007	-0.080
Plant height	-0.105	0.301	0.263	0.016	-0.081
Primary branches per plant	0.141	0.221	-0.022	0.351	0.484
Clusters per plant	0.376	0.121	-0.051	-0.030	0.149
Pods per cluster	0.289	0.067	-0.252	-0.170	-0.131
Pods per plant	0.401	0.134	-0.155	-0.094	0.086
Pod length	0.192	0.074	0.581	0.056	-0.098
Seeds per pod	0.230	0.175	0.487	-0.054	-0.015
Pod yield per plant	0.408	0.168	-0.050	-0.007	-0.084
100-seeds weight	0.068	-0.065	0.226	0.452	-0.359
Days to maturity	-0.089	0.430	-0.228	0.179	0.006
Seed yield per plant	0.396	0.164	-0.041	0.004	-0.126
Iron content	-0.014	-0.091	0.040	0.583	0.458
Zinc content	-0.187	0.082	0.341	-0.198	0.095
Protein content	-0.020	0.056	0.172	-0.463	0.563
Eigen value	5.009	2.337	1.608	1.234	1.086
Proportion of variation (%)	31.3	14.6	10.1	7.7	6.8
Cumulative variance (%)	31.3	45.9	56.0	63.7	70.5



(a) Variability Observed for flower colour



(b) Variability Observed for leaf shape

Supplementary Fig. 1. Variability Observed for flower colour (a); (b) Variability Observed for leaf shape

Indian J. Plant Genet. Resour. 35(2): 185–188 (2022)
DOI 10.5958/0976-1926.2022.00019.5

RESEARCH ARTICLE

Studies on Variability and Correlation in Bael (*Aegle marmelos* (L.) Correa)

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(Received: 04 November, 2020; Revised: 23 June, 2022; Accepted: 11 July, 2022)

Bael is an underutilized, indigenous and designated as vulnerable species. The pilgrimage centre Shri Kalmurudeshwaraswamy matha of Sakharayapattana in Chikkamagaluru district in Karnataka, India has a sacred groove of bael trees. Due to natural sexual propagation they have acquired a high level of variability. Hence, the documentation and conservation of this groove is essential. A total of 356 trees of bael were evaluated for growth, fruit characters and showed considerable variations. Among 356 trees, SB-353 was found superior with respect to fruit characters *viz*., length (13 cm), volume (310 cm^3), weight (68.03 g), pulp weigh (202.40 g) and higher pupl:seed (10.22). For stability, the plus tree has to be evaluated for future usage in bael improvement.

Key Words: Bael, Correlation studies, Fruit morphological parameters, Tree morphological parameters

Introduction

Bael (*Aegle marmelos* (L) Correa) is one of the important underutilized, indigenous fruit crops of India, known for its high medicinal and nutritional values. It was found wild in the Sub-Himalayan tract and dry deciduous forests of Central and Southern India. Therefore, a large number of landraces are available in different diversity regions. Each tree is genetically diverse from others as most of them are of seedling origin. Individual tree varies greatly in their morphology; variation may be due to genetical or environmental or both. This heterozygous nature gives scope for further selection and the establishment of desirable trees. Information on magnitude of variation and association of characters is useful as a basis for the selection of desirable types. Hence, identification of elite trees for the region is necessary for promoting its production, productivity and quality of the fruits under the Southern Transitional Zone of Karnataka, India. Keeping in view the above facts investigation was conducted to characterize the bael trees for morphological, yield and yield attributing traits.

Material and Methods

The survey was conducted during 2018-19 in the Sakharayapattana, Chikkamagaluru district of Karnataka which is located between 13.431757° N latitude and 75.924549° E longitude with an elevation of 763 m above mean sea level. The experimental site is located in Central Dry Agro-climatic Zone of Karnataka with red sandy loam soils. The observations were recorded based on guidelines by PPV and FR. The colour of leaves was distinguished using colour chart of Royal Horticulture Society. The age of the trees were ranged between 20-40 years and grown widely. The height of trees was measured using Ravi altimeter. The data recorded during the investigation of morphological variations among 356 trees were analyzed using frequency distribution. Among 356 trees, 76 fruit bearing trees were subjected to fruit yield and quality parameters. These were grouped into 18 clusters based on cluster analysis (using SPSS software). Using cluster membership, superior 12 trees were selected and subjected to one way ANOVA (Gomez and Gomez, 1984). The correlation co efficient analysis was done using standard formulae described by Gomez and Gomez, 1984.

Results and Discussion

For selection of a superior tree it's important to study the vigour nature of tree. Tree height also represents the vigour nature of the trees. Maximum height (9 m) of the tree was observed in three trees SB-8, SB-9, SB-354 and it was minimum (4 m) in ten trees (SB-33, SB-35,

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SB-38, SB-39, SB-40, SB-41, SB-42, SB-44, SB-146 and SB-351).

Upright growth and spreading growth habit were observed in 274 trees 82 trees respectively. The variation in tree height and plant growth habit might be due to its inherent genetic character and existing climatic condition. These lines are under conformity with the results of Gupta and Mishra (2002), Rai *et al*. (2002) and Bhawna and Mishra (2011). Grey bark colour was observed in maximum trees (171). However, Greyish black colour was observed in a minimum number of trees (25) and other bark colours *viz*., greyish brown (97), brown (35), black (28) was also observed among the trees. Such variations in bark colour among the trees may be governed by it's a varietal expression and existing favourable environmental condition. Among the trees surveyed, a higher number of trees (275) showed irregular bark splitting habit and a lesser number (81) of trees showed rectangular bark splitting habit. It's directly influences the easy flow of phloem transportation of tree. The variation in bark splitting nature might be due to genotypic and phenotypic character associated with the trees. The observations are in agreement with the result of Parihar and Pandey (2019). Regarding leaf base cunate, round and tapering leaf base were observed in 217,7 and 132 trees respectively. With respect to leaf shape lanceolate (345 trees), broadly ovate shape (1 tree), ovate (8 trees) and lanceolate to ovate (2 trees) were recorded. The maximum number of trees (252) showed the crenulated type of leaf margin whereas; the minimum number of trees (104) recorded the crenate type of leaf margin. A greater variation in leaf base, leaf shape and leaf margin among the trees might be due to the presence of abundant genetic variability associated with their trees. Such variation may be due to its better photosynthetic activity and their utilization for building up of new cells and also might be due to the genomic constitution of individual tree. Such variation in leaf shape was previously reported by Saroj *et al.* (2008).

Bael trees observed significant differences in leaf colour Yellow-green leaves were observed in 185 trees whereas, green leaves were observed in 171 trees. The difference in leaf colour may be due to it's inherent genetic character and also due to physiological and metabolic changes in chlorophyll and photosynthetic activity involved in the colour variation of the leaf. No variability was observed for thorniness. Thorn length had a significant variation which was ranged from 3.2 to 6 cm. The variation in thorn length might be due to genotypic and phenotypic character associated with the trees. The result was in agreement with the previous author Parihar and Pandey (2019).

Variation in pomological characters is furnished in Table. 1. The length of fruit showed a significant variation on bael trees which was ranged from 6.50 cm to 13 cm. The longest length of fruit (13 cm) was observed in tree SB-353 which was statistically on par with SB-73 (12 cm) and a shorter length of fruit was recorded in tree SB-305 (6.50 cm). The variation in fruit length might be governed by genomic character associated with the trees and also favourable climatic condition. The results are in agreement with the findings of Kaushik *et al.* (2000), Nath *et al*. (2003) and Pandey *et al*. (2013). Maximum fruit volume was observed in tree SB-353 (310 cm^3) followed by trees SB-253 and SB-209 (160 cm^3 respectively) however, minimum was observed in tree SB-305. The variation in fruit volume due to positive interrelationship with fruit weight and also governed by its varietal expression of individual tree. The findings are in agreement by previous authors Lal (2002), Rai *et al.* (2002) and Singh and Mishra (2010). Fruit weight was ranged from 68.30g to 320g. Maximum weight (320 g) of fruit was registered in SB-353 tree followed by SB-209 and SB-187 (169 g) whereas, minimum fruit weight was observed in SB-305 (68.30 g). The difference in fruit weight attributed to an increase in fruit length, fruit volume, pulp weight, seed weight, skull weight of the fruits of the trees. The findings are also in agreement with the results of Ram and Singh (2003), Srivastava and Singh (2004) and Mitra *et al* (2010). Significantly maximum pulp weight was observed in tree SB-353 (202.40 g) and minimum was observed in tree SB-80 (27.70 g). An increase in pulp weight could have positively associated with higher fruit weight and it's negatively associated with seed weight. A variation in pulp content could be due to inheriting genetic makeup of the trees. The results are in agreement with the findings of Nidhi and Gehlot (2007). The highest number of locules per fruit was observed in SB-187 (10.67) whereas; the lowest was recorded in SB-73 (7.67). The difference in a number of locules per fruit might be due to varietal character associated with the trees. Similar results have been reported by Misra *et al* (2000) and Pandey *et al* (2008). Maximum skull thickness (6.76 mm) in SB-203 tree followed by SB-305 (6.50 mm) however, minimum skull thickness was

Table 1. Variability in pomological characters of bael tree

Tree	Fruit length (cm)	Fruit volume (cm^3)	Fruit weight (g)	Pulp weight per fruit (g)	No. locules per fruit	No. of seeds per locule	Skull thickness (mm)	No. of seeds per fruit	Seed weight per fruit (g)	Seed : Pulp	Pulp : Seed
SB-353	13.00 a	310.00 a	320.00 a	202.40 a	10.66 a	3.75 bc	4.95 c	40.00 b	19.80 b	0.10 h	10.22 a
SB-323	9.00 bc	130.00 c	138.50 d	70.50 c	8.33 c	3.96 b	4.29 d	33.00 cd	15.20 c	0.22 f	4.62 g
SB-305	6.50 d	60.00 f	68.30 g	51.50 g	8.67 c	2.54 cd	6.50 a	22.00 f	10.10 g	0.20 f	5.10 e
SB-275	7.50 cd	110.00 d	119.00 e	58.10 f	8.00 c	3.88 b	3.20 e	31.00 d	15.50 c	0.27 d	3.75 i
SB-297	7.20 d	110.00 d	122.00 e	60.98 e	9.00 b	2.11 d	4.50 cd	19.00 f	9.50 g	0.16 g	6.42 c
SB-253	10.00 b	160.00 b	155.70 c	75.59 b	8.00 c	3.13 c	5.67 b	25.00 ef	12.50 f	0.17 g	6.05 d
SB-209	8.50 c	160.00 b	169.00 b	74.30 b	9.67 b	6.93 a	3.89 d	67.00 a	39.57 a	0.53 a	1.88 l
SB-203	7.30 d	140.00 c	151.50 c	57.69 f	10.00 a	3.50 b	6.76 a	35.00 c	17.88 c	0.31 c	3.23 j
SB-187	7.30 d	160.00 b	169.00 b	57.69 f	10.67 a	2.72 c	5.91 b	29.00 de	14.50 d	0.25 e	3.98 h
SB-80	9.00 bc	80.00 e	85.90 f	27.70 i	9.00 b	2.89 c	5.50 b	26.00 e	13.22 ef	0.48 b	2.10 k
SB-123	9.00 bc	90.00 e	96.20 f	30.31 h	9.00 b	0.89 e	6.30 ab	8.00 g	3.07 h	0.10 h	9.87 b
SB-73	12.00 a	140.00 c	158.60 b	65.50 d	7.67 d	3.39 bc	4.89 c	26.00 e	13.50 e	0.21 f	4.85 f
F value	**	**	**	**	**	**	**	**	**	**	**
S.Em ±	0.35	5.77	4.31	0.82	0.32	0.17	0.17	1.05	0.27	0.01	0.08
CD @ 5%	1.01	16.85	12.58	2.38	0.93	0.51	0.50	3.08	0.79	0.02	0.22

** Significant at 5% and 1

observed in tree SB-275 (3.20 mm). Variation in skull thickness was noticed due to the distinct nature of the tree. The findings are also in agreement with the results of Mitra *et al.* (2010). Significantly minimum number of seeds per locule was observed in the SB-123 tree (0.89) and maximum number of seeds per locule was observed in the SB-209 tree (6.93). The decrease in seed number per locule has a positive correlation with higher pulp content. A similar variation in seed number per locule could be attributed to the inherited genetic makeup of the trees. Findings are in agreement with the results of Singh and Mishra (2010). Significantly maximum seed weight per fruit was observed in SB-209 (39.57 g) followed by SB-353 (19.80 g) and minimum seed weight per fruit was observed in SB-123 (3.07 g). The difference in seed weight was attributed to difference in the number and size of seeds among the trees. It was observed that the ratio of seed to pulp was maximum in SB-209 (0.53) followed by tree SB-80 (0.48) and it was found minimum in SB-353 and SB-123 (0.10 respectively). Pulp to seed ratio was found maximum in tree SB-353 (10.22) which was followed by SB-123 (9.87) and the minimum was observed in SB-209 (1.88) tree. These observations are in agreement with similar results have been reported by Nidhi and Gehlot (2007). An increase in pulp weight could have positively associated with higher fruit weight and it's negatively associated with seed weight.

Correlation studies

Correlation coefficient analysis measures the mutual relationship between various plant characters and determines the component characters on which, selection can be based. Correlation provides information on the nature and extent of relationship between all pairs of characters. Fruit length showed positively significant association with fruit volume, fruit weight and pulp weight. while with number of seeds per locule, number of seeds per fruit, seed weight per fruit showed positive non-significant association. Number of locules per fruit and skull thickness showed negative non-significant association. Fruit volume showed highly significant positive association with the characters like fruit weight and pulp weight per fruit. Whereas, characters like number of locules per fruit, number of locules per fruit, number of seeds per fruit and seed weight per fruit showed non-significant positive association with fruit volume. Skull thickness showed a negative non-significant association with fruit volume. Fruit weight

showed highly significant positive relation with pulp weight per fruit whereas for other parameters it showed a non significant relation. Number of locules per fruit showed non-significant association with all characters. It was observed highly significant positive association between number of seeds per locule and number of seeds per fruit, seed weight per fruit. Skull thickness showed a non significant negative association with for all characters except for number of locules per fruit it showed positive non significant association. Number of seeds per fruit showed highly significant positive association with seed weight per fruit. The results are also in-line with Divakara (2008).

Conclusion

Variations are recorded for all the 356 trees of bael for growth and fruit characters. It is concluded that among the 356 trees the tree SB-353 was found superior with respect to fruit characters (fruit weight, fruit length, fruit volume, pulp weight). Therefore, tree SB-353 to be evaluated further for stability and if found consistent can be used in the bael improvement programme.

Acknowledgement

Authors are hearty thankful to the Director of Research, Directorate of research, University of Agricultural and Horticultural Sciences, Shivamogga for providing necessary facilities.

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Indian J. Plant Genet. Resour. 35(2): 189–193 (2022)
DOI 10.5958/0976-1926.2022.00020.1

RESEARCH ARTICLE

Assessment of Morphological Characterization and Genetic Variability of Mandukaparni (*Centella asiatica* L.) Accessions

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(Received: 27 January, 2021; Revised: 17 March, 2022; Accepted: 02 May, 2022)

Centella asiatica L., commonly known as Indian pennywort is one of the chief herbs having a lot of medicinal properties and is used extensively both in traditional and modern medicine. However, the unrestricted exploitation coupled with the limited cultivation has threatened their survival and is now included in the list of endangered species. In the present study, fifteen accessions of *Centella asiatica* have been assessed for the morphological characterization and genetic variability to identify superior genotypes. Genetic variability parameters (phenotypic coefficient of variation, genotypic coefficient of variation, heritability, and the genetic advance) have been evaluated using ANOVA for twelve morphological characters such as shoot length, leaf length, leaf width, rosette diameter, petiole length, fresh leaf weight, and dry leaf weight, etc. Genetic divergence using D^2 analysis has been performed by dividing the accessions into five clusters based on the morphological characters. The results showed that the maximum intra-cluster distance has been found in cluster IV (24.45) followed by cluster I (22.13) and cluster III (21.67), whereas the maximum inter-cluster distance has been found between clusters IV and III (40.45) followed by cluster V and IV (36.81). Thus, the germplasm which includes clusters III and IV can be used in crop improvement using breeding programs.

Key Words: *Centella asiatica* L., Genetic variability, GCV, PCV, Yield

Introduction

Centella asiatica L. is a faintly aromatic creeping evergreen in tropical and subtropical countries growing in swampy areas, including parts of India, Pakistan, Sri Lanka, Madagascar, South Africa, South Pacific, and Eastern Europe. It is an important medicinal herb used in both traditional Chinese medicine and Indian Ayurvedic medicine for thousands of years. It is one of the chief herbs for treating skin problems, healing wounds, and revitalizing the nerves and brain cells, hence primarily known as a "brain food" (Singh *et al.,* 2010). It is effective in the treatment of stomach ulcers, digestive disorders, mental fatigue, diarrhea, epilepsy, hepatitis, syphilis, and asthma (Goldstein and Goldstein, 2012), enhancing memory and longevity (Subathra *et al.,* 2005; Singh *et al.,* 2008), blood purifier (Anjana and Jha, 2008). In the light of its versatile medicinal properties, the pharmaceutical industry's demand for *Centella asiatica* has been sharply increasing thus leading to the overexploitation of the plant, which may have led to losses of genetic diversity and elite accessions. Moreover, it has been listed as threatened plant species by the International Union for Conservation of Nature and Natural Resources (IUCN) and also as an endangered species (Sharma and Kumar, 1998).

It is one of the important medicinal plants in the International market of medicinal plant trades. However, large-scale collection from wild habitats increases the threat of genetic erosion and does not result in the quality of the raw material. Therefore, there is a need to evolve high-yielding cultivars for commercial cultivation. Variability present in the natural wild populations of *Centella asiatica* offers ample scope for a breeder to select and identify new superior accessions in terms of more growth, yield, and quality for commercial cultivation. Selection based on only phenotypic differences will often mislead the breeder as it is influenced by the environment on the expression of traits. Hence, the knowledge of genetic parameters such as genotypic coefficient of variation, heritability, genetic advance as percent of the mean (GAM) is vital to judging the best genotype. The best genotype should

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possess a high heritability of desired characters coupled with a high genetic advance. For the improvement of any trait, the information on its association with other traits is very crucial because selection for a particular trait invariably affects its associated traits. Morphological characterization is considered to be an important first step in the description and classification of germplasm since the breeding program in any crop is mainly based on the magnitude of genetic variability (Smith and Smith, 1989). Genetic variability for agronomic traits as well as quality tests is important in all crop improvement programmes since this component is transmitted to the next generation (Singh *et al.,* 1996; Bhandari *et al.,* 2017). In the present study, an attempt has been made to identify the better performance genotypes among the given accessions in terms of morphological growth, yield, and vegetative traits of *Centella asiatica* crop.

Materials and Methods

The present study was conducted at ICAR-Indian Institute of Horticultural Research (IIHR), Hessaraghatta, Bengaluru-89 during *Kharif* 2015. The size of the plot was 6m × 1.2 m and the germplasms were planted in a randomized block design and replicated three times at a spacing of 30 cm × 10 cm. The material comprised 15 accessions (Table 1) along with one check variety Vallabh Medha, procured from different regions of the state, India, and maintained the germplasm at ICAR-IIHR. Observations were recorded from five randomly selected plants of each germplasm and in each replication for twelve traits: shoot length (cm), number of primary branches per plant, number of leaves per plant, leaf length (cm), leaf width (cm), number of nodes per plant, rosette diameter (cm), petiole length (cm), specific leaf weight (g/cm^2), internodal length (cm), fresh leaf yield per hectare and dry leaf yield per hectare.

Statistical analysis of parameters

The collected data has been subjected to analysis of variance (ANOVA) by making use of means of replication, as suggested by Goulden (1959) and the test of significance was worked out by referring to the standard "F" table suggested by Snedecor (1967). The GCV and PCV were calculated as per the method suggested by Burton and Devane in 1953 and were classified as suggested by Sivasubramaniam and Madhava 1973. Heritability (h^2) in a broad sense was calculated as per the formula suggested by Allard 1960 and expressed in percent as Low (<30%), moderate (30-60%), and high (>60%). The genetic advance as percent mean was categorized into low (<10), moderate (10-20), and high (>20) as per the formula suggested by Johnson *et al*. (1955). The genetic diversity was assessed by using Mahalanobis D^2 statistics 1936 and the grouping of accessions into clusters using Tocher's method, as suggested by Rao 1952.

Table 1. List of germplasm used in the experimental study

S.No.	Germplasm	Place of collection	State
1.	Vallabh Medha (Check)	DMAPR, Anand	Gujarat
2.	IIHR CA-1	Jalgaon	Maharashtra
3.	IIHR CA-2	Bengaluru	Karnataka
4.	IIHR CA-4	Jalgaon	Maharashtra
5.	IIHR CA-5	Mangalore	Karnataka
6.	IIHR CA-6	Jabalpur	Madhya Pradesh
7.	IIHR CA-7	Jabalpur	Madhya Pradesh
8.	IIHR CA-8	Khasi hills	Meghalaya
9.	IIHR CA-9	Khanapur	Karnataka
10.	IIHR CA-10	Khanapur	Karnataka
11.	IIHR CA-11	Honnavar	Karnataka
12.	IIHR CA-12	Gonikoppal	Karnataka
13.	IIHR CA-13	Shivamogga	Karnataka
14.	IIHR CA-14	Polyploid-1 (IIHR)	Karnataka
15.	IIHR CA-15	Polyploid-2 (IIHR)	Karnataka

Results and Discussion

The knowledge of genetic variation is important for selection in crop improvement programmes and the success of any crop improvement programme is dependent not only on the amount of genetic variability present in the population but also on the extent to which it is heritable, which sets the limit of progress that can be achieved through selection. From the analysis of variance, it was observed that the genotypes varied significantly for all the twelve characters studied which are presented in Table 2. The mean squares due to genotypes were highly significant for all the traits indicating thereby the presence of genetic variability in the experimental material. The analysis of variance revealed the existence of significant differences among the germplasm studied for most of the traits indicating the presence of a considerable amount of variability. Hence there is a scope for the selection of potential genotypes for the breeding program.

Table 2. Analysis of variance for the 12 characters of *Centella asiatica* L.

Source of variation (df)	Mean sum of squares											
	SL (cm)	NPB (no.)	NN (no.)	NL (no.)	LL (cm)	LW (cm)	IL (cm)	RD (cm)	PL (cm)	SLW ($m^2.kg^{-1}$)	FLW ($q.h^{-1}$)	DLW ($q.h^{-1}$)
Replication (2)	1.177	23.402	17.276	469.242	0.043	0.004	0.116	0.863	0.625	0.001	130.609	3.825
Genotype (14)**	25.708	12.568	17.390	216.679	1.280	4.896	4.738	31.712	19.172	0.060	67.219	2.626
Error (28)	0.722	1.612	1.836	21.629	0.047	0.042	0.092	0.446	0.629	0.000	5.847	0.167
CV (%)	9.914	8.213	9.085	6.917	8.029	4.690	4.313	5.658	9.800	7.390	17.115	15.429
GCV (%)	33.66	12.36	15.26	11.99	23.63	29.15	17.71	27.36	30.73	36.22	33.48	35.03
PCV (%)	35.09	14.84	17.76	13.85	24.96	29.52	18.23	27.95	32.25	73.15	33.66	35.78
(h^2)	92.02	69.37	73.84	75.04	89.65	97.47	94.4	95.9	90.79	24.52	98.88	95.84
GA	66.52	21.21	27.02	21.4	46.1	59.28	35.45	55.21	60.31	36.94	68.57	70.64

** significant at P<0.01 SL= Shoot length; NPB= No. of primary branches/plant; NN= No. of nodes/plant; NL= No. of leaves/plant; LL= Leaf length; LW= Leaf weight; IL= Internodal length; RD= Rosette diameter; PL= Petiole length; SLW= Specific leaf weight; FLW= Fresh leaf weight (q/ha); DLW= Dry leaf weight (q/ha).

The evaluation of genetic variation parameters, viz., PCV, GCV, heritability, and genetic advance as percent of the mean for different characters. In this study, the germplasm revealed a significant amount of variability for all the twelve traits studied. Statistically, the GCV for all characters studied was lesser than PCV suggesting that the variability is due to an interaction with the environment up to some extent (Pushpa *et al.,* 2013). The difference between GCV and PCV was observed to be highest in specific leaf weight as it is strongly influenced by the environment. Low GCV and PCV were found for most of the characters indicating lesser environmental influence in the expression of a particular character (Verma *et al.,* 2014). Specific leaf weight, leaf dry weight, shoot length, petiole length, leaf width, rosette diameter, and leaf length were recorded with high GCV and PCV. It was indicated that these traits were governed by additive gene effects with low environmental effects. A similar result was obtained for shoot length in *Andrographis paniculata* (Misra *et al.,* 2001). Moderate GCV and PCV were recorded for the number of primary branches, number of nodes, number of leaves, and internodes length. A similar result was found for primary branches in sweet basil (*Ocimum basilicum*) (Ibrahim *et al.,* 2013).

The coefficient of variation indicates only the extent of variability present for different characters and does not consider the heritable portion. The heritability estimates devoid of environmental influence from the total variability indicate the accuracy with which superior segregants in a population can be selected by their phenotypic performance, thus making the selection more effective. However, heritability estimates itself is not an indication of the amount of genotypic progress that would result from selecting the superior segregants (Johnson *et al.,* 1955). Genetic advance is an important selection parameter that helps plant breeders in the selection of elite germplasm from a genetically diverse population. Estimates of heritability with genetic advances are more reliable and meaningful than individual consideration of the parameters (Nwangburuka and Denton, 2012). Therefore, heritability estimates along with high genetic advances are more useful criteria in predicting the resultant effects for selecting the best individual. This is since a character may have very high heritability but very less phenotypic variation gives rise to the very low genetic gain. Most of all the characters recorded high heritability along with genetic advance as percent of mean except specific leaf weight with low heritability (24.52%). Higher heritability indicated that these characters were less influenced by the environment and direct selection for these traits would be effective for further improvement (Abou El-Nasr *et al.,* 2013). The traits such as shoot length, leaf length, leaf width, rosette diameter, petiole length, fresh leaf weight, and dry leaf weight recorded high GCV, PCV, heritability, and genetic advance as percent of the mean. A similar report was found in stevia for leaf yield per plant (Gaurav *et al.,* 2008). Low heritability was observed for specific leaf weight which may be due to the character being highly influenced by environmental effects and genetic improvement through selection will be difficult due to

masking effects of the environment on the genotypic effects. It can be concluded that traits such as shoot length, leaf length, leaf width, rosette diameter, petiole length, fresh leaf weight, and dry leaf weight had higher GCV, PCV, heritability, and genetic advance as percent of the mean are agreeable for the selection and can be effectively used for genetic improvement of breeding programs.

In the present study, D^2 analysis was carried out on fifteen germplasm using twelve characters showed significant variability for all characters evaluated and were further confirmed through the pattern of distribution of fifteen *Centella asiatica* germplasm into five clusters. Among five clusters, cluster I had six germplasm and formed the largest cluster followed by clusters III and IV had three germplasm in each cluster, cluster II with two germplasm and cluster V was unique, as it had only one germplasm which indicates a wider divergence among the germplasm. In Mahalanobis D^2 analysis, among six different clusters, the maximum intracluster distance was shown by cluster IV (24.45), followed by cluster I (22.13) and cluster III (21.67), whereas the inter-cluster distance was found between cluster IV and III (40.45) followed by cluster V and IV (36.81). It showed that germplasm which includes clusters III (IIHR CA-7, IIHR CA-14, and IIHR CA-15) and IV (IIHR CA-8, IIHR CA-9, and IIHR CA-11) can be used in hybridization programmes to generate a wide range of transgressive segregants in population for developing new varieties.

Conclusion

The present investigation provides that *Centella asiatica* germplasm such as IIHR CA-7, IIHR CA-14, and IIHR CA-15 and IIHR CA-8, IIHR CA-9, and IIHR CA-11, are genetically diverse by cluster distance and it can be concluded that there is a wider variability among these promising germplasms of *Centella asiatica* in terms of morphological characters which can be used in future crop improvement work.

Acknowledgment

Authors are grateful to the ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru-Karnataka, India for providing the necessary facilities and support to carry out this study.

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Indian J. Plant Genet. Resour. 35(2): 194–198 (2022)
DOI 10.5958/0976-1926.2022.00021.3

RESEARCH ARTICLE

Early Growth and Yield Performance at Nursery Stage of a Set of Brazilian Wild *Hevea* Germplasm of IRRDB Collection

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(Received: 08 April, 2021; Revised: 28 June, 2022; Accepted: 02 July, 2022)

A set of 55 wild *Hevea brasilensis* accessions from three provenances of Brazil *viz.,* Acre (AC), Rondonia (RO) and Mato Grosso (MT), along with two popularly cultivated clones RRII 105 and RRIM 600 were evaluated for their early growth and yield performance in the first six years of growth in a nursery in the traditional rubber growing region of Kerala state, India. Relatively high clonal variability was observed for yield, while variation for girth, girth increment, single leaf area, total number of laticifer rows, diameter of latex vessels, bark thickness and crotch height ranged from medium to low. The yield ranged from 0.04 g/t/t (RO 5358) to 11.19 g/t/t (RO 5018), bark thickness from 1 mm (AC 5896) to 5.30 mm (MT 4771), total number of laticifer rows from 3 (MT 5824) to 10.67 (RO 2841) and diameter of latex vessels from 10.69 µm (AC 5487) to 21.66 µm (MT 4762). Girth ranged from 10.75 cm (AC 5896) to 35.60 cm (RO 5432) in the 6^{th} year, while girth increment over three years ranged from 0.50 cm/year (AC 5466) to 5.47 cm/year (RO 5432), crotch height ranged from 1.88 m (RO 5364) to 5.14 m (MT 4690) and single leaf area from 37.51 cm^2 (RO 5318) to 150.40 cm^2 (RO 5365). The accessions were ranked using all the above parameters except single leaf area, for overall performance. Rank sum values ranged from 30 to 340 with a general mean of 194.33. Based on this study, the top 20% of the potential accessions showing early growth vigour and yield were identified which could be of use in broadening the narrow genetic base of currently cultivated genotypes and for future crop improvement programmes.

Key Words: *Hevea brasiliensis,* Performance index, Variation, Wild germplasm

Introduction

Although *Hevea* genus has 11 species, *Hevea brasiliensis* is the popularly cultivated species for its natural rubber production in the world. Currently cultivated popular clones were derived from the Wickham collection comprised of a very small gene pool, since it was collected from a limited area of native Amazon region, Brazil (Schultes, 1977). The intensive directional selection over the years for yield alone has further narrowed the genetic base (Wycherley, 1969), and has further resulted in a slowdown in genetic advances in recent breeding phases (Tan, 1981; Seguin *et al*., 1995; Simmonds, 1989). Due to human interventions, genetic resources of *Hevea* are fast depleting in the primary center of origin. The need of *Hevea* conservation was felt by the natural rubber industry, thus the International Rubber Research and Development Board (IRRDB) had organized an expedition during 1981 in the primary centre of origin of the *Hevea* crop, the Amazon basin, covering three states, Acre (AC), Mato Grosso (MT) and Rondonia (RO) in Brazil. This resulted in collection of over 60,000 seeds and budwood from 194 exceptionally good trees (Ong *et al*., 1983). These accessions were distributed to IRRDB member countries, and those received in India, are being conserved in conservation cum source bush nurseries.

As part of evaluation of this germplasm, data on various growth parameters and juvenile yield characters were recorded in the early growth phase. The present study was undertaken to ascertain the extent of genetic variability in the population and identify potential accessions for using in the breeding programmes.

Materials and Methods

The study was conducted at the Central Experiment Station of the Rubber Research Institute of India, Chethackal, Kerala state, India. A nursery was laid out in an augmented block design during 2001, to evaluate the *Hevea* germplasm for various characteristics. A total of fifty five wild *Hevea brasiliensis* accessions from three provenances of Brazil *i.e*., Acre (11), Mato Grosso (33) and Rondonia (11), along with two popular clones *viz*.,

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RRII 105 and RRIM 600 were included in this study**.** The spacing adopted was 1 × 1 m with five plants per plot (Fig. 1). The recommended cultural practices of Rubber Board were followed.

The characters studied include test tap yield (g/t/t) at a height of 30 cm above the bud union, bark thickness (mm), total number of laticifer rows (TLVR), diameter (µm) of latex vessels and growth characters *viz*., girth (cm) of the stem at a height of 30 cm above the bud union from the third to sixth year after planting and average girth increment (cm/year) over three years, calculated using the girth data of 3rd year to 6th year. Crotch height (m) was recorded as the height from the bud union to the first branching level and single leaf area (cm^2) measured using leaf area meter. All the plants were test tapped at the age of 5 years following ½S d/3 (half spiral, once in three days) system of tapping. The data were subjected to analysis of variance for augmented design (Petersen, 1994). Performance of all these genotypes were assessed by rank sum method (Kang, 1988).

Results and Discussion

The range and general mean values of nine characters in comparison with the two control clones is presented in Table 1. Relatively high clonal variability was observed for yield, while variation for girth, girth increment, single leaf area, total number of laticifer rows, diameter of latex vessels, bark thickness and crotch height ranged from medium to low. The yield per tree per tap (g/tree/tap) ranged from 0.04 g/t/t (RO 5358) to 11.19 g/t/t (RO 5018) with a general mean of 1.17 g. Accession RO 5018 was the highest yielder followed by RO 2841 (8.71 g/t/t), RO 5432 (7.86 g/t/t), MT 4762 (2.79 g/t/t), MT 4885 (2.66 g/t/t) and AC 5817 (1.64 g/t/t),

Fig. 1. A view of *Hevea* germplasm nursery at RRII, India

Table 1. Variability for yield, yield components and growth related characters in wild *Hevea* germplasm

Characters	Wild accessions			Control clones		CV (%)
	Minimum	Maximum	General mean	RRII 105	RRIM 600	
Yield (g/t/t)	0.04 (RO 5358)	11.19 (RO 5018)	1.17	2.88	5.82	41.97
Bark thickness (mm)	1.00 (AC 5896)	5.30 (MT4771)	2.76	3.17	3.08	15.14
Total number of laticifer rows	3.00 (MT 5824)	10.67 (RO2841)	6.39	9.74	8.41	21.98
Diameter (µm) of latex vessels	10.69 (AC 5487)	21.66 (MT4762)	16.74	17.19	18.42	25.23
Girth (cm) after 6th year of planting	10.75 (AC5896)	35.60 (RO5432)	20.62	21.56	20.54	20.91
Girth increment (cm/yr) over 3 years	0.50 (AC5466)	5.47 (RO 5432)	2.35	2.47	2.69	22.45
Crotch height (m)	1.88 (RO 5364)	5.14 (MT 4690)	3.04	2.65	2.46	14.09
Single leaf area (cm^2)	37.51(RO5318)	150.40 (RO 5365)	69.34	52.68	56.46	22.93

Note: Figures in parenthesis denotes the name of accession.

where as the control clone RRII 105 and RRIM 600 recorded 2.88 g/t/t and 5.82 g/t/t, respectively. Yield components such as bark thickness ranged from 1 mm (AC 5896) to 5.30 mm (MT 4771), total number of laticifer rows from 3 (MT 5824) to 10.67 (RO 2841), diameter of latex vessels from 10.69 µm (AC 5487) to 21.66 µm (MT 4762). Studies by Reghu *et al*. (2008). Annamma *et al*. (1989), Mercy *et al*. (1995), Rao *et al.* (1999 & 2011) and Abraham *et al*. (2002) have also reported wide variability in the wild *Hevea* germplasm with respect to certain yield and growth traits in traditional rubber growing region in India.

Girth has been identified as one of the most important traits contributing for latex yield in *Hevea* (Ho *et al*., 1973; Narayanan *et al*., 1974; Hamzah and Gomez, 1982). Girth of the plants ranged from 10.75 cm (AC 5896) to 35.60 cm (RO 5432) and girth increment over three years ranged from 0.50 cm/yr (AC 5466) to 5.47 cm/yr (RO 5432). Highest girth was recorded in the clones RO 5432 (35.60 cm), MT 4906 (31.63 cm) and MT 6021 (27.90 cm). Girth increment was the highest in RO 5432 (5.47 cm/yr), followed by RO 2841 (4.25 cm/yr), AC 4677 (3.68 cm/yr) and MT 4707 (3.67 cm/yr). Growth vigour is genetically controlled and there is marked clonal variation with regard to girth increment under tapping and its effect on yield (Ferwerda, 1969). Genotypes with early growth vigour are highly useful for reducing immaturity period.

The crotch height showed a range of 1.88 m (RO 5364) to 5.14 m (MT 4690). MT 4690 registered the highest crotch height followed by RO 5442 (5.05 m) and MT 4772 (4.36 m). Moderate estimates of coefficients of variation for certain growth and yield characters in wild *Hevea* germplasm was reported by Rao *et al*. (1999, 2006 & 2011) and Abraham *et al*. (2002). Wide range of variability was observed for the trait single leaf area which ranged from 37.51 cm^2 (RO 5318) to 150.40 cm^2 (RO 5365) with a general mean of 69.34 cm^2. Accession RO 5365 (150.40 cm^2), AC 5497 (108.20 cm^2) and RO 5350 (96.70 cm^2) recorded higher leaf area.

Even though the general mean values of the hybrid controls were higher than those of the wild accessions for all traits except crotch height and single leaf area, certain individual wild accessions showed high mean values for yield and growth characters particularly in RO 5018, RO 2841 and RO 5432 for yield, MT 4771, RO 5432 and MT 4906 for bark thickness, MT 4762, RO 5432 and MT 6021 for total number of laticifer rows, MT 4762, RO 5432 and MT 6021 for diameter of latex vessels, RO 5432, MT 4906 and MT 6021 for girth, RO 5432, RO 2841 and AC 4677 for girth increment, MT 4690, RO 5442 and MT 4772 for crotch height, AC 2719, RO 5365 and AC 5497 for single leaf area. Accessions superior to the control clone RRII 105 for yield and growth characters are shown in Table 2. These genotypes with high girth and girth increment coupled with higher crotch height and yield gives an early indication of fast growth of these wild germplasm which is useful in reducing the immaturity period. Moreover, those genotypes with high crotch height coupled with good girth also indicate their high timber potential. Chapuset *et al*. (1995) reported variation in branching behavior among the wild germplasm. Branching habit in rubber tree was a clonal character and many clones were found to branch at a higher levels in the plantations of Malaysia (MRB, 2003). The tendency of wild *Hevea* germplasm to branch at a higher level than the Wickham clones was also reported by Azwar *et al*. (1995) and Rao *et al*. (1999 2006 and 2011).

Table 2. Accessions superior to the control clone RRII 105 for yield and growth related characters

Characters	No.	Accessions	RRII 105	RRIM 600	CD (5%)
Yield (g/t/t)	3	RO 5018, RO 2841, RO 5432	2.88	5.82	3.79
Bark thickness (mm)	18	(Top fifteen only) MT4771, RO5432, MT4906, MT6051, MT4839, RO2841, RO5002, MT4762, MT6020, MT6021, MT5095, AC4677, MT5117, MT4707, RO5018.	3.17	3.08	0.98
Total number of laticifer rows	3	RO 2841, MT 6020, MT 4762	9.74	8.41	NS
Diameter (μm) of latex vessels	29	(Top fifteen only) MT4762, RO5432, MT6021, RO2841, MT4757, AC5896, AC6134, MT5940, MT4885, AC5688, MT4771, MT5117, MT4796, MT5924, MT5993.	17.19	18.42	NS
Girth (cm) after 6th year of planting	25	(Top fifteen only) RO5432, MT4906, MT6021, AC5688, MT6051, RO2841, MT4839, RO5002, MT4707, AC4677, MT5095, AC4816, MT4785, AC5497, MT5122.	21.56	20.54	9.14
Girth increment (cm/yr)	25	(Top fifteen only) RO5432, RO2841, AC4677, MT4707, MT6021, MT5924, MT4771, MT4906, RO5002, AC5688, AC4816, MT6048, MT4762, MT5940, AC5497.	2.47	2.69	1.20
Crotch height (m)	34	(Top fifteen only) MT4690, RO5442, MT4772, MT6051, MT5122, MT5095, RO5413, AC4677, MT4694, AC5904, MT5086, MT4771, MT4757, MT6007, AC5487.	2.65	2.46	0.75
Single leaf area (cm^2)	43	(Top fifteen only) RO5365, AC5497, RO5350, AC5904, MT5993, MT5152, MT4694, RO5002, AC5487, MT4772, RO5358, MT5951, MT4796, MT5117, MT5095.	52.68	56.46	26.00

NS- not significant.

In order to identify genotypes with maximum number of desirable attributes, the performance of each of the eight characters was pooled using rank sum method. The ranking of each genotype based on parametric relationship of yield and yield components- bark thickness, total number of laticifer rows, diameter of latex vessels and growth characters such as girth, girth increment and crotch height are shown in Table 3.

The rank sum values ranged from 30 to 340 with a general mean of 194.33. High rank was recorded in the genotype RO 5432 (340) followed by RO 2841(322), MT 4906 (320), MT 6021 (310), AC 5688 (306) and MT 4762 (291). These genotypes showed relatively high yield and vigorous growth. The wild accessions MT 5824 (77), RO 5365 (74) and AC 5466 (30) recorded the lowest rank sum value. Balasimha *et al*. (1988), Mercy (2001) and Rao *et al*. (2006) also reported similar ranking in cocoa and wild *Hevea* accessions, respectively while evaluating the genotypes. Top 20 per cent of the potential genotypes identified as best performers are RO 5432 (340), RO 2841 (322), MT 4906 (320), MT 6021 (310), AC 5688 (306), MT 4762 (291), MT 4771 (278), AC 4677 (275), RO 5018 (274), MT 6051 (272), RO 5002 (271), MT 4757 (269) and MT 5095 (264).

Various morphological, anatomical, biochemical and physiological characters of the rubber tree are ultimately manifested in the volume of latex obtained by tapping and the quantum of rubber it contains. A vigorous habit in the early growth phase of the plant reduces the immaturity period. In general, yield and vigour in *Hevea brasiliensis* are hardly separable (Simmonds, 1989). The present study resulted in the identification of vigorous accessions with wide variability for growth and yield

Table 3. Top ranking of wild accessions based on yield and growth parameters

Accession	Rank sum	Rank
RO 5432	340	1
RO 2841	322	2
MT 4906	320	3
MT 6021	310	4
AC 5688	306	5
MT 4762	291	6
MT 4771	278	7
AC 4677	275	8
RO 5018	274	9
MT 6051	272	10
RO 5002	271	11
MT 4757	269	12
MT 5095	264	13
MT 4707	253	14
AC 4816	251	15
MT 4885	251	15
MT 4839	239	17
MT 6048	237	18

General mean = 194.33

traits. Certain genotypes showed superiority for growth and yield characters which indicated its early vigour and yield.

Wide variability was observed for various growth and yield contributing traits. RO 5018, RO 2841, RO 5432, MT 4762, MT 4885, AC 5817, AC 5688, AC 4816, AC 6134, MT 4906, MT 4771, AC 4677, MT 6051, RO 5002, MT 4757, MT 5095 and MT 4707 and were identified as vigorous genotypes with high growth and yield in the juvenile phase which will be useful for reducing the immaturity period and have potential value for timber. These selections could be further evaluated and incorporated in breeding programmes for evolving new rubber clones.

Acknowledgements

The author is grateful to P Aneesh (Assistant Statistician) for help in analysis of the data.

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Indian J. Plant Genet. Resour. 35(2): 199–208 (2022)
DOI 10.5958/0976-1926.2022.00022.5

RESEARCH ARTICLE

Expedition Collection, Characterization and Diversity Analysis of the New Wild Sugarcane Germplasm from Manipur

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(Received: 03 August, 2021; Revised: 11 April, 2022; Accepted: 16 April, 2022)

Saccharum spontaneum, a wild relative of sugarcane had contributed significantly for introgressing wider adaptability, high tillering, resistance to biotic and abiotic stresses in the commercial sugarcane varieties. Manipur state representing tropical to sub-alpine climatic condition was explored and 61 *S. spontaneum*, 4 *S. officinarum*, 1 *Erianthus fulvus* and 1 *Narenga fallax* accessions were collected. *S. spontaneum* had wider distribution and were found in river bank to filed bunds, isolated clumps to large population. High variability was observed for many quantitative characters including plant height, tillering, stalk diameter and internode length. Clustering analysis separated the collection into 6 major clusters. While *Erianthus fulvus* and *S. officinarum* formed individual clusters, the *S. spontaneum* accessions were separated into 4 clusters mostly based on biomass potential, plant height and morphotypes. Genetic diversity and utilization of these clones in improving low temperature tolerance, biomass improvement and widening genetic base of sugarcane varieties are discussed.

Key Words: Clusters, *Erianthus*, Germplasm collection, *Narenga*, North East India, *Saccharum spontaneum*

Introduction

Plant genetic resources play a major role in crop improvement programmes not only for improving yield and quality but also for imparting resistance to pests and diseases and tolerance to abiotic stresses. Sugarcane is one of the classical examples wherein wild species have significantly contributed for the development of new varieties with high cane yield and wider adaptability (Panje, 1972; Giamalva *et al.,* 1984; Berding and Roach, 1987, Govindaraj *et al.,* 2005). *Saccharum officinarum* (L.) was the cultivated cane in tropical countries including India and *Saccharum barberi* (Jeswiet) was the main sugarcane species under cultivation in northern India before the introduction of man-made sugarcane hybrids. Area under *S. officinarum* varieties could not be expanded in India because they essentially required well managed condition due to lesser adaptability to varied agro-climatic conditions and susceptibility to biotic and abiotic stresses although they had thick and tall canes and high juice sucrose content. However major breakthrough in sugarcane varietal development was achieved in 1918 when the first interspecific hybrid (ISH) between *S. officinarum* (Vellai: 2n=80) and *S. spontaneum* (Coimbatore local: 2n=64) was made and first-generation interspecific hybrid Co 205 with wider adaptability was released for cultivation in subtropical India. This was the first interspecific hybrid involving a wild species which became commercially successful without resorting to back crosses for eliminating the undesirable genes from wild species which was mainly attributed to the 2n+n transmission of gametes ensuring the full complement of *S. officinarum* in the F_1 hybrid.

After the successful introduction of Co 205 for commercial cultivation, several ISH hybrids and their backcrosses with either *S. officinarum* or near commercial 'Co' canes were developed for further improving cane yield, juice quality and wider adaptability, a process called nobilization. Introduction of array of varieties developed through nobilization process occupied sizable areas in both tropical and subtropical India thus totally replacing the traditional varieties. The success of these varieties was primarily due to high tillering, wider adaptability to various agro-climatic conditions and resistance to biotic and abiotic stresses contributed by *S. spontaneum* (D'Hont *et al.,* 1996; Terajima *et al.,* 2007; Wang *et al.,* 2008; da-Silva 2017; Govindaraj and Mahadevaswamy 2021). Other species /genera of *Saccharum* complex viz., *S. robustum*, *S. barberi* and *Erianthus* were also

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introduced later in breeding programmes but their contribution to varietal development was relatively very less (Govindaraj *et al.,* 2012). Genome analysis of the modern sugarcane varieties also indicated around 15% of the sugarcane genome was contributed by *S. spontaneum* genome (D'Hont *et al.,* 1995). Red rot, a major disease is practically managed with the introgression of *S. spontaneum* as vertical resistance was positively and significantly correlated with the number of *S. spontaneum* chromosomes present in their genome (Natarajan *et al.,* 2001). At present it is also used for transferring cold tolerance (Hale *et al*., 2014), high biomass energycane development (Govindaraj 2017; Govindaraj, 2020), winter hardiness and winter ratoonability and improving vegetation and restoration programmes (Pandey *et al.,* 2015). As the ISH or *S. spontaneum* introgressed clones showed higher fibre, lignocellulose or higher biomass (Govindaraj *et al*., 2014; Cosentino *et al.,* 2015; Terajima *et al.,* 2007; Govindaraj and Nair, 2014), there was renewed interest among sugarcane breeders in utilizing *S. spontaneum* and *Erianthus arundinaceus* in the development of energycanes and multipurpose sugarcane varieties (Govindaraj *et al.,* 2012).

For the successful introgression programme, new germplasm carrying new genes /allelic variation for agronomic and stress tolerance traits should be included. Hence, several explorations were conducted by the ICAR-Sugarcane Breeding Institute, Coimbatore for the collection of new *S. spontaneum* and other species of *Saccharum* complex in India and other countries (Nair *et al.,* 1991; Nair *et al.,* 1993; Nair *et al.,* 2006; Abraham *et al.,* 2008; Nair and Sekaran, 2009; Govindaraj *et al.,* 2014; Govindaraj *et al.,* 2016; Karthigeyan *et al.,* 2020; Govindaraj and Mahadevaswamy, 2021; Govindaraj and Mahadevaswamy, 2021a). However, a part of North Eastern Region of India especially the Manipur State which still conserves rich source of biodiversity due to less human interference was not explored so far, hence an expedition was carried out in the state to collect new variability found in *Saccharum* complex.

Materials and Methods

Manipur state of India was explored for the collection of wild sugarcane germplasm during 2011 by a team of scientists consisting of Dr. VA Amalaraj and Dr. P Govindaraj, Principal Scientists, ICAR-Sugarcane Breeding Institute, Coimbatore. The state had two distinct physiography – the hills and valley and flat plain and representative locations of these geography were covered during the expedition. The expedition route was prepared and 10 major districts viz., West Imphal, Ukhrul, Thoubal, Tamenglong, Senapati, Kangpoki, East Imphal, Churachandpur, Chandal and Bishnupur were explored (Fig. 1). A minimum of 5km distance was maintained between the adjacent collections to avoid duplications. Visits were made to home gardens where cultivated *S. officinarum* were grown either for chewing or pooja purposes. While *S. spontaneum* and *E. fulvus* accessions were collected as clumps with germinating underground stolon, *S. officinarum* and *Narenga fallax* were collected as stem cuttings with active buds. Data on the latitude, longitude and altitude of the collection site were recorded. Quantitative traits such as plant height (cm), leaf length (cm), leaf breadth (cm), stalk diameter (cm), arrow length (cm), peduncle length and intermodal length were recorded *in situ* (Table 1) in three replicates for further analysis. Variability statistics such as range, mean and SD were worked out to understand the variation among the collections (Table 2). Genetic diversity was estimated through hierarchical clustering with the morphological data recorded on new accessions. Jaccard's coefficient of similarity was calculated (Rohlf, 2002) by the following equation:

$$GSij = Nij /[Ni + Nj+ Nij]$$

where

Nij is the total number of attributes common to both accessions i and j, and Ni and Nj are the number of attributes only present in accession i and j, respectively.

Fig. 1. Exploration route for the collection of wild sugarcane germplasm covering different districts of Manipur

Table 1. Location, species status and quantitative traits recorded at the collection site of the new germplasm collected from Manipur

S. No	Accession No	Place of collection	Species status	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Arrow length (cm)	Peduncle length (cm)	Stalk diameter (cm)	Internode length (cm)
1	IND 11-1657	Ukhrul	EF	140	100	1.7	34	42	–	–
2	IND 11-1680	Chandal	NF	154	101	0.7	88	77	0.5	7.1
3	IND 11-1671	Thoubal	SO	400	145	5.0	–	–	3.7	18.0
4	IND 11-1672	Thoubal	SO	450	166	9.0	–	–	5.4	19.0
5	IND 11-1684	Thoubal	SO	300	140	6.0	–	–	4.1	14.0
6	IND 11-1703	West Imphal	SO	320	160	5.0	–	–	3.8	13.0
7	IND 11-1637	Bisnupur	SS	130	79	0.5	30	40	0.5	10.5
8	IND 11-1638	Bisnupur	SS	400	102	0.7	50	43	0.8	14.0
9	IND 11-1639	Churachandpur	SS	400	132	0.5	55	32	0.4	13.0
10	IND 11-1640	Churachandpur	SS	275	112	0.4	45	61	0.6	16.0
11	IND 11-1641	Churachandpur	SS	310	93	0.6	60	72	1.8	14.0
12	IND 11-1642	Churachandpur	SS	310	124	0.4	52	55	0.8	13.0
13	IND 11-1643	Bisnupur	SS	600	119	3.8	60	60	2.3	25.0
14	IND 11-1644	Bisnupur	SS	320	114	3.8	55	53	2.2	16.0
15	IND 11-1645	Bisnupur	SS	100	57	0.6	27	42	0.5	13.0
16	IND 11-1646	Bisnupur	SS	200	93	0.6	34	31	0.5	8.0
17	IND 11-1647	West Imphal	SS	190	112	1.0	32	30	0.5	14.0
18	IND 11-1648	Senapati	SS	185	103	0.7	50	64	0.5	21.0
19	IND 11-1649	Senapati	SS	200	120	1.5	49	61	0.7	11.0
20	IND 11-1650	Senapati	SS	215	129	2.6	57	58	1.1	12.0
21	IND 11-1651	Tamenglong	SS	260	110	0.8	50	55	0.5	17.0
22	IND 11-1652	Tamenglong	SS	250	120	0.3	40	44	0.5	9.0
23	IND 11-1653	Tamenglong	SS	210	125	0.7	63	53	0.9	8.0
24	IND 11-1654	Tamenglong	SS	160	65	0.3	36	60	0.4	7.5
25	IND 11-1655	Tamenglong	SS	200	118	0.5	25	28	0.7	9.5
26	IND 11-1656	West Imphal	SS	180	93	0.4	22	34	0.9	24.0
27	IND 11-1658	Ukhrul	SS	180	95	1.3	65	59	0.8	13.0
28	IND 11-1659	Ukhrul	SS	170	82	0.6	40	55	0.7	11.0
29	IND 11-1660	Ukhrul	SS	175	71	0.8	27	42	0.7	14.0
30	IND 11-1661	Ukhrul	SS	210	81	0.4	42	54	0.6	13.0
31	IND 11-1662	Ukhrul	SS	130	73	0.7	38	39	0.9	14.0
32	IND 11-1663	Ukhrul	SS	120	62	0.4	30	36	0.5	12.0
33	IND 11-1664	Ukhrul	SS	122	104	0.2	45	59	0.5	10.0
34	IND 11-1665	Ukhrul	SS	215	101	0.2	37	55	0.5	5.6
35	IND 11-1666	Ukhrul	SS	120	79	0.2	33	53	0.4	8.0
36	IND 11-1667	Senapati	SS	124	77	0.2	79	74	0.6	14.0
37	IND 11-1668	Senapati	SS	380	95	1.2	25	43	0.6	8.0
38	IND 11-1669	Thoubal	SS	140	120	1.1	30	48	0.9	25.0
39	IND 11-1670	Thoubal	SS	180	76	0.3	40	53	0.7	11.0
40	IND 11-1673	Thoubal	SS	260	94	0.6	59	77	0.6	7.5
41	IND 11-1674	Thoubal	SS	130	131	1.0	50	62	1.1	7.5
42	IND 11-1675	Thoubal	SS	210	145	2.0	40	65	0.8	8.5
43	IND 11-1676	Thoubal	SS	480	115	3.7	42	39	2.1	30.0
44	IND 11-1677	Thoubal	SS	190	105	0.5	57	65	0.5	6.5
45	IND 11-1678	Chandal	SS	200	106	0.3	40	52	0.6	7.5
46	IND 11-1679	Chandal	SS	167	126	0.3	41	40	0.5	13.0
47	IND 11-1681	Chandal	SS	200	101	0.6	57	62	0.6	8.0
48	IND 11-1682	Chandal	SS	198	48	0.2	29	48	0.6	9.5
49	IND 11-1683	Chandal	SS	380	109	1.0	59	72	1.1	28.0
50	IND 11-1685	Thoubal	SS	350	93	1.9	56	64	1.4	12.5
51	IND 11-1686	Thoubal	SS	270	144	2.0	46	44	0.8	11.9
52	IND 11-1687	Thoubal	SS	209	116	0.9	46	54	0.7	7.0

S. No	Accession No	Place of collection	Species status	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Arrow length (cm)	Peduncle length (cm)	Stalk diameter (cm)	Internode length (cm)
53	IND 11-1688	East Imphal	SS	105	130	0.6	55	50	0.8	13.0
54	IND 11-1689	East Imphal	SS	200	124	0.2	55	67	0.5	10.0
55	IND 11-1690	East Imphal	SS	160	114	0.5	30	42	0.5	8.5
56	IND 11-1691	East Imphal	SS	290	132	1.3	48	61	1.9	32.0
57	IND 11-1692	East Imphal	SS	80	24	0.1	27	33	0.4	4.5
58	IND 11-1693	East Imphal	SS	170	96	0.2	39	55	0.5	12.5
59	IND 11-1694	West Imphal	SS	380	126	0.3	54	60	0.9	21.5
60	IND 11-1695	West Imphal	SS	220	95	0.3	50	46	0.6	14.0
61	IND 11-1696	West Imphal	SS	200	121	0.4	49	52	0.5	20.5
62	IND 11-1697	West Imphal	SS	120	59	0.4	39	51	0.2	8.0
63	IND 11-1698	West Imphal	SS	150	68	0.3	21	35	0.1	14.0
64	IND 11-1699	West Imphal	SS	260	89	0.2	48	57	0.5	10.0
65	IND 11-1700	Kangpokpi	SS	210	126	0.9	20	24	0.9	13.5
66	IND 11-1701	Kangpokpi	SS	230	121	2.0	47	39	0.8	12.0
67	IND 11-1702	West Imphal	SS	130	103	0.2	31	39	0.5	5.5

EF: *Erianthus fulvus* NF: *Narenga fallax* SO: *S. officinarum* SS: *S. spontaneum*

Table 2. Descriptive statistics on the seven quantitative traits among the new *S. spontaneum* accessions

	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Arrow length (cm)	Peduncle length (cm)	Stalk diameter (cm)	Internode length (cm)
S. officinarum							
Mean	367.50	152.75	6.25	–	–	4.25	16.00
Maximum	450.00	166.00	9.00	–	–	5.40	19.00
Minimum	300.00	140.00	5.00	–	–	3.70	13.00
SD	69.94	12.26	1.89	–	–	0.79	2.94
S. spontaneum							
Mean	221.48	101.59	0.84	43.57	50.84	0.76	12.97
Maximum	600.00	145.00	3.80	79.00	77.00	2.30	32.00
Minimum	80.00	24.00	0.10	20.00	24.00	0.10	4.50
SD	98.43	24.82	0.86	12.63	12.30	0.45	6.03
Erianthus fulvus							
Mean	140	100	1.7	34	42	–	–
Narenga fallax							
Mean	154	101	0.7	88	77	0.5	7.1

Dendrogram based on similarity coefficients were generated through NTSYS-PC by using unweighted pair group method with arithmetic averages (UPGMA).

Results and Discussion

Distribution of Saccharum species complex in Manipur

Manipur is blessed with an amazing variety of flora and fauna and 67% of the geographical area is hill tract covered forests. Depending on the altitude of hill ranges, the climatic condition varies from tropical to sub-alpine. The wet forests and the pine forests occur between 900-2700 m above mean sea level (AMSL) and they together sustain a host of rare and endemic plants. Exploration was conducted in ten districts of Manipur namely West Imphal, Ukhrul, Thoubal, Tamenglong, Senapati, Kangpoki, East Imphal, Churachandpur, Chandal and Bishnupur and 67 new accessions were collected which included 61 *S. spontaneum*, 4 *S. officinarum* (Thoubal and West Imphal districts), 1 *Erianthus fulvus* (Ukhrul district) and 1 *Narenga fallax* (Chandal district) accessions. Distribution of species was not even across the state while *S. spontaneum* was found abundant and could be collected from all the districts, *E. fulvus*, *N. fallax* and *S officinarum* were restricted to certain areas.

Earlier expeditions in different states of India also indicated the wider adaptability of *S. spontaneum* under

drought (Govindaraj *et al.,* 2014), water logging and salinity (Govindaraj and Mahadevaswamy, 2021), low temperature (Govindaraj and Mahadevaswamy, 2021a) and hilly slopes (Govindaraj *et al.,* 2016). During the exploration various forms of *S. spontaneum* could be collected from river banks (IND 11-1639, IND 11-1651, IND 11-1653, IND 11-1654, IND 11-1665, IND 11-1682, IND 11-1702), irrigation channels (IND 11-1670, IND 11-1681, IND 11-1686), bunds of paddy fields (IND 11-1646, IND 11-1659, IND 11-1692), small water ponds (IND 11-1663, IND 11-1656, IND 11-1677), hilly slopes (IND 11-1648, IND 11-1655, IND 11-1664, IND 11-1688), rocky crevices (IND 11-1640) and forest areas (IND 11-1666). Distribution of the species in different geo-agro-climatic conditions indicated its wider adaptability as reported earlier by Govindaraj and Mahadevaswamy (2021) and Govindaraj *et al*. (2014). A possible reason attributed for the wider distribution of *S. spontaneum* could be its mode of reproduction. The underground rhizomes are carried away by rivers, river fed canals, irrigation channels, ponds and the true seed called, fluff dispersed through wind and scattered over dry land, waste lands, hilly slopes, rocky crevices and forest areas can germinate under favourable conditions and establishes in different ecological niches (Govindaraj and Mahadevaswamy, 2021a).

During the exploration we could found establishment of *S. spontaneum* as individual clump (IND 11-1652, IND 11-1659, IND 11-1670, IND 11-1692), small groups (IND 11-1650, IND 11-1693, IND 11-1694) and very large populations (IND 11-1651, IND 11-1653, IND 11-1681, IND 11-1682, IND 11-1695) (Fig. 2). All the accessions were in flowering stage during our exploration (September and October 2011) and three accessions namely IND 11-1646, IND 11-1658 and IND 11-1686 were early in flowering and the fluff started shedding at the time of collection.

Four *S. officinarum* (IND 11-1671, IND 11-1672, IND 11-1684, IND 11-1703) were collected from the home gardens in Thoubal (3) and West Imphal (1) districts. A splendid accession (IND 11-1672) was collected from Thoubal district with 4.5 m cane height and 5.4 cm cane diameter (Fig. 4). The new accessions were very tall with high biomass and more juice volume hence can be used in the breeding programme after induction of flowering for not only incorporating these useful traits but also broadening the genetic base of the new sugarcane varieties (Govindaraj *et al.,* 2021).

Fig. 2. Large population of *S. spontaneum* (IND 11-1682) spotted in the river bank at Chandel district

Variability in the new collections

High variation was found among *S. spontaneum* collections for tillering ability. A high proportion of accessions collected were high tillering (IND 11-1639, IND 11-1651, IND 11-1673) while very few were low in tillering (IND 11-1640, IND 11-1649, IND 11-1699). The high tillering accessions could be utilised in the breeding programmes after characterization to improve the genetic base of the modern sugarcane varieties (Sindhu *et al.,* 2011; Govindaraj *et al.,* 2011).

The accession, IND 11-1637 was collected from fallow land in West Imphal district. This was a short plant with short leaf blade (79 cm), leaf width (0.5 cm) and thin stalks (0.5 cm) (Table 1). The tallest accession (IND 11-1643 – 600 cm) was collected from a small pond fed with perennial water source (Fig. 3). It had broader leaves (3.8 cm), thicker stalk (2.3 cm diameter) and longer internodes (25 cm). IND 11-1676 (480 cm), IND 11-1638 (400 cm), IND 11-1639 (400 cm), IND 11-1668 (380 cm) and IND 11-1683 (380 cm) were the next best accessions for plant height and all these accessions could be potential parents for breeding for biomass improvement. Very short accession IND 11-1692 (80 cm) was spotted in a paddy field bund. It had very narrow leaf (0.1 cm) with very short arrow (27 cm) and peduncle (33 cm). Very large population (IND 11-1638) was spotted on the river bank of Khuga river while another large population (IND 11-1639) was found along irrigation channels in Bhisnapur and Chrurachandpur, respectively. IND 11-1665 and IND 11-1682 were the two representative samples collected from the large population of *S. spontaneum* found in

Fig. 3. IND 11-1643 – the tallest collection (600 cm) of *S. spontaneum* from a small pond with perennial water source in Bisnapur district

Fig. 4. Thick (5.4 cm) and tall (450 cm) *S. officinarum* (IND 11-1672) collected from a home garden in Thoubal district

Ukhrul and Chandal districts, respectively and both were medium in height and with internode length of 9.5 cm each. A heavy tillering type IND 11-1651 with more than 100 tillers per clump was collected along the Barak river bank in Tamenglong district which receives an average of 3330 mm annual rainfall. The longest internodes were observed in IND 11-1691 (32 cm) and IND 11-1676 (30 cm) and the shortest internode was noticed in IND 11-1692 (4.5 cm). Long internodes are the indication of faster growth which is one of the important selection criteria in sugarcane varietal development programme (Lingle and Tew, 2008). While IND 11-1667 (79 cm) and IND 11-1673 (77 cm) recorded the longest arrow and peduncle length respectively, IND 11-1700 showed the shortest arrow (20 cm) and peduncle (24 cm) length. High variability for morphological traits (Govindaraj *et al.,* 2014; Govindaraj *et al.,* 2016; Karthigeyan *et al.,* 2020) and molecular markers (Alwala *et al.,* 2006; Govindaraj *et al.,* 2011; Sindhu *et al.,* 2011; Govindaraj *et al.,* 2021) among the *S. spontaneum* accessions were reported earlier. The new collections have unique germplasm with tall growing and high biomass *S. officinarum* and high tillering and long internodes *S. spontaneum* and will add more variability and diversity to the world collection of sugarcane germplasm maintained at ICAR - Sugarcane Breeding Institute, India.

Among the ten districts, two districts namely Kangpokpi and part of Ukhrul were situated in the high attitude hilly areas and other districts were in the plains. Ten accessions were collected from Tamenglong followed by 9 each in West Imphal and Ukhrul districts. IND 11-1700 and IND 11-1701 were collected from high altitude Kangpokpi district (Table 3). Both the accessions were robust in stature with high mean values for leaf length (123.5 cm), leaf width (1.45 cm) and stalk diameter (0.85 cm) but reduced arrow (33.5 cm) and peduncle (31.5 cm) lengths (Table 4). Six accessions namely IND 11-1658 (1876 m AMSL), IND 11-1659 (1210 m AMSL), IND 11-1660 (1202 m AMSL), IND 11-1661 (1300 m AMSL), IND 11-1662 (1457 m AMSL) and IND 11-1663 (1053 m AMSL) were located in hilly areas experiencing low temperature during winter and the other collections were from the plain areas. All the accessions were medium statured with average stalk diameter (0.62 cm). All these high-altitude accessions can be screened for cold tolerance for using in breeding programme for transferring winter ratoonability in sugarcane. Churachandpur had the lowest collection of

4 accessions and all were tall with mean plant height of 323.75 cm and high mean leaf length (115.25 cm) and stalk diameter (0.9 cm). Six collections were made from Bishanpur district and all the collections were tall with the mean plant height of 291.67 cm, leaves width of 1.67 cm, cane diameter of 1.13 cm and internode length of 14.42 cm. From East Imphal, six more accessions were collected which were short in nature with reduced leaf width (0.48 cm) and 5 accessions each were collected from Tamenglong, Senapati and Chandal.

One *E. fulvus* (IND 11-1657) was collected in the hilly slopes in the high altitude Ukhrul district. IND 11-1657 occurred as small population in an abandoned field and was in flowering stage. It was a medium tall plant (140 cm) with short leaf and medium leaf width (1.7 cm). Among the species of *Erianthus*, *E. arundinaceus* is the only cane forming species while other species produce stalks only during flowering to bear the inflorescence called arrow. Hence, in the sugarcane improvement programmes mostly *E. arundinaceu*s was used as parent for introgressing high biomass, more tillering and pests and disease resistance (Govindaraj, 2020). Recently non-cane forming species like *E. procerus* was also used for incorporation of biomass, drought tolerance and red rot resistance (Nair *et al.,* 2017, Mohanraj *et al.,* 2019). The new accession of *E. fulvus* is a high altitude (1879 m AMSL) collection and supposed to possess low temperature tolerance hence can be crossed with commercial and near commercial canes for improving winter ratoonability after screening under cold condition.

Narenga fallax is another genera in the *Saccharum* complex which can be hybridized with the *Saccharum* species including commercial canes. IND 11-1680 was the *N. fallax* accession collected from the fallow land in Chandal district. This was found as an isolated clump with heavy tillering with the height of 154 cm, leaf length of 101 cm, leaf width of 0.7 cm and stalk diameter of 0.5 cm. Flowering was almost completed with the

Table 3. Distribution of *S. spontaneum* and other species in different districts of Manipur

District	*S. spontaneum*	*S. officinarum*	*E. fulvus*	*Narenga fallax*
West Imphal	9	1	–	–
Ukhrul	9	–	1	–
Thoubal	10	3	–	–
Tamenglong	5	–	–	–
Senapati	5	–	–	–
Kangpokpi	2	–	–	–
East Imphal	6	–	–	–
Churachandpur	4	–	–	–
Chandal	5	–	–	1
Bishnupur	6	–	–	–
Total	61	4	1	1

Table 4. Variation in the mean performance of the *S. spontaneum* accession collected from different districts

Districts	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Arrow length (cm)	Peduncle length (cm)	Stalk diameter (cm)	Internode length (cm)
West Imphal	203.33	96.22	0.39	38.44	44.89	0.52	14.61
Ukhrul	160.22	83.11	0.53	39.67	50.22	0.62	11.18
Thoubal	241.90	113.90	1.40	46.60	57.10	0.96	12.74
Tamenglong	216.00	107.60	0.52	42.80	48.00	0.60	10.20
Senapati	220.8	104.8	1.24	52.00	60.00	0.70	13.2
Kangpokpi	220.00	123.50	1.45	33.50	31.50	0.85	12.75
East Imphal	167.50	103.33	0.48	42.33	51.33	0.77	13.42
Churachandpur	323.75	115.25	0.48	53.00	55.00	0.90	14.00
Chandal	229.00	98.00	0.48	45.20	54.80	0.68	13.20
Bishnupur	291.67	94.00	1.67	42.67	44.83	1.13	14.42

arrow and peduncle of 88 cm and 77 cm respectively. This accession can be used in hybridisation for the broadening the genetic base as intergeneric hybrids involving *Narenga* were reported earlier (Chang *et al.,* 2020).

Genetic diversity among 67 accessions

Sugarcane is a cross pollinated crop and high genetic diversity was reported earlier due to complex genome structure, polyaneuploidy, di or tri species origin and high heterozygosity (D'Hont *et al.,* 1996; Grivet and Arruda 2002; Hemaprabha *et al.,* 2006; Govindaraj *et al.,* 2012; Saravanakumar *et al.,* 2014; Senthilkumar *et al.,* 2014). New accessions collected from Manipur were also analysed for variability and clustering pattern. Hierarchal clustering established six major groups with 1, 4, 1, 6, 1 and 54 accessions in different groups. Cluster I had the single *E. fulvus* accession IND 11-1657. Cluster II grouped all four *S. officinarum* collected from Thoubal and West Imphal districts. This species had long and thick canes and presence of 3 rows of root eyes in the nodal region. Mean stalk diameter (4.25 cm) was the highest among the clusters and it also showed the longest (152.75 cm) and the widest (6.25 cm) leaves. Cluster III had only IND 11-1643, a *S. spontaneum* accession which was the tallest plant (600 cm). This was unique among the accessions with the highest arrow length (60 cm), peduncle length (60 cm) and internode length (25 cm). Among the 61 *S. spontaneum* accessions collected, IND 11-1643 recorded the highest leaf width (3.80 cm) and stalk diameter (2.3 cm) also. It clearly indicated that morphological traits have greater power in grouping of genotypes based on variability present (Govindaraj *et al.*, 2016). Cluster IV had 6 accessions (IND 11-1638, IND 11-1639, IND 11-1668, IND 11-1676, IND 11-1683, IND 11-1694) which were distributed in six different districts and all showed robust growth and high biomass with more than 380 cm plant height. The cluster IV had average plant height of 403.33 cm, leaf width of 1.23 cm and internode length of 19.08 cm. Accessions from this group can be used in the breeding programme for incorporating early growth and long internodes. Another small cluster V had only one accession of *S. spontaneum* (IND 11-1692) which was the shortest plant collected during the entire exploration. It recorded only 80 cm plant height with short (24 cm), very narrow (0.1 cm width) leaves, thin canes (0.40 cm stalk diameter) and short internodes (4.5 cm). Earlier reports revealed shorter plants among 2n=40 cytotype (Sobhakumari *et al.,* 2013). Hence, this accession is expected to have the basic cytotype (2n=40) which should be confirmed through cytological analysis.

The biggest cluster VI had 53 *S. spontaneum* and 1 *Narenga fallax* accessions representing all the 10 districts. Most of the districts in Manipur have overlapping agro-climatic conditions which may be one of the reasons for non-separation of accessions according to their location of collections. The average plant height, stalk diameter, internode length, leaf length and leaf width were 195.63 cm, 0.71 cm, 13.09 cm, 101.41 cm and 0.75 cm, respectively. All the *S. spontaneum* accessions were grouped in this cluster except the extreme accessions for plant height (IND 11-1692, IND 11-1643) and vigorous and high biomass (IND 11-1638, IND 11-1639, IND 11-1668, IND 11-1676, IND 11-1683, IND 11-1694). This largest cluster was subgrouped into two clusters namely VIa and VIb with 12 and 42 accessions respectively. Critical analysis of the pattern of sub grouping indicated that accessions in subgroup VIa were tall (282.08 cm) with thick canes (1.03 cm), broad leaf (1.19) and long internodes (18.66 cm) compared to the mean of the cluster VIb. *S. spontaneum* collected from Maharashtra (Govindaraj *et al.,* 2012, Gujarat (Govindaraj *et al.,* 2014), Assam (Govindaraj and Mahadevaswamy 2021a), Punjab and Haryana (Karthigeyan *et al.,* 2020) and Western Ghats (Govindaraj *et al.,* 2021a) also showed high genetic variability. *Narenga fallax* is the member of *Saccharum* complex and is crossable with the *Saccharum* species and possesses useful genes for tolerance to drought and water logging and resistance to pest, diseases and root parasites. Grassl (1977) reported successful intergeneric hybrids involving *Narenga fallax* and development of few hybrids. However, more efforts are required in utilising this genus in the regular breeding programme by overcoming the barriers in crossing and development of progenies for introduding new useful genes.

In general, clusters were formed as per their morphotypes. The new collections with high diversity will add to the genetic diversity of the gene pool maintained at ICAR-Sugarcane Breeding Institute, Coimbatore. The accessions collected from the high altitude and low temperature regions can be screened for low temperature tolerance and exploited in breeding for better winter ratoonability in the subtropical regions. New *S. officinarum* collections with high biomass production can be also used in the genetic enhancement programmes for improving cane yield and diversifying the genetic base of the modern sugarcane varieties.

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Indian J. Plant Genet. Resour. 35(2): 209–216 (2022)
DOI 10.5958/0976-1926.2022.00023.7

RESEARCH ARTICLE

Genetic Analysis of Polygenic Traits for Seed, Fibre and Dual Purpose Linseed (*Linum usitatissimum* L.) Genotypes Grown under Sub Temperate Conditions of Western Himalayas

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(Received: 02 September, 2021; Revised: 20 May, 2022; Accepted: 27 May, 2022)

Genetic analysis of linseed plant genetic resources is very important to understand gene action and combining ability for crop improvement. Eight genetically diverse lines of linseed namely Surbhi, Bhagsu, Nagarkot, T-397, Himani, Baner, JRF-1 and JRF-4 were evaluated through half diallel cross analysis under sub temperate conditions of Western Himalayas. These eight parental lines identified for seed, fibre and dual purpose trait and 28 F_1 progenieswere grown in CRBD to identify potential parents and their cross combinations for yield and associated traits under variable environments. Significant level of genetic variability was observed among studied genotypes for the yield, fibre and their contributing traits. Genotype×environment (G×E) interaction was also significant except for plant height and technical height indicating a definite role of environment in the performance of genotypes. Non-additive gene action was recorded for maximum traits. Based on GCA, three genotypes *viz*., Baner, Nagarkot and Surbhi were recorded to be good general combiner for seed yield whereas genotypes, JRF-1 and JRF-4 were good for early maturity and fibre yield. These promising genotype harbor the genes for various traits like high seed and fibre yield, early maturity, rust and powdery mildew tolerance. On the basis of high SCA effect, four cross combinations *viz*., Himani×JRF-4, T-397×JRF-4, Bhagsu×Nagarkot and Surbhi×Baner were found best for seed yield while four other *viz*., Surbhi×T-397, Nagarkot×Himani, Surbhi×Baner and Surbhi×Bhagsu were found best for fibre yield in pooled over environments. These genotypes and cross combinations can be used in further hybridization and selection can be done for early maturity, disease tolerance, seed, fibre and dual purpose trait as per the need of linseed breeding programmes in the country.

Key Words: Combining ability, G×E interaction, Gene action, *Linum usitatissimum*

Introduction

Linseed (*Linum usitatissimum* L.) is an annual, self-pollinated, diploid (2x=2n=30) oilseed belonging to the family Linaceae (Singh *et al*., 2021). The species is believed to have originated in regions of the east Mediterranean Sea (Vavilov, 1951). Linseed occupies an important place among oilseed as it is highly nutritive and every portion of it is used commercially (Sran *et al*., 2021). Higher availability of an essential polyunsaturated Omega-3 and Omega-6 fatty acids makes linseed oil unique (Gill, 1987; Reddy *et al*., 2013; You *et al*., 2018; Walkowiak *et al*., 2022). The fibre extracted from straw is utilized to make strong yarns, such as, linen fabrics, sewing strings,carpets and curtains while the coarser grades are used for making twines, canvas bags and quality papers (Jhala and Hall, 2010). In India, the linseed productivity is very low (567 kg/ha) as compared to that of the world (1005.90 kg/ha) (FAO Stat, 2017). The main encumbrances behind low production are the crop cultivation mainly on marginal or sub-marginal soils under rainfed and input-starved conditions. The lower productivity can also be attributed to non-availability of high yielding varieties for routine cultivation (Kiran *et al*., 2012). Development of high yielding varieties along with earliness is always prioritized by the breeders. In linseed, the major breeding goals for seed purpose involves improving seed yield, oil content and its quality and for flax purpose objectives mainly includes higher technical height and quality of fibre (Zare *et al*., 2021).

In order to amalgamate desirable attributes along with high yield, the pertinent approach is recombinant breeding. Thereby, choice of the parents having abundant potential for hybridization is one of the critical tasks. Selection of appropriate parents helps in production of superior recombinant genotypes (Bertan *et al*., 2007). Mostly parents are selected on the basis of *per se*

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performance, adaptation and diversity, but such criteria do not give desired results because the ability of the parents to combine properly with each other dependson the complex interactions among genes (Allard, 1960). Therefore, the selection of best parents for hybridization must be based upon the knowledge of the combining ability of parents. Some genotypes have the ability to combine with number of other lines indicating good general combining ability (GCA) whereas some combine well only in few cross combinations suggesting specific general combining ability (SCA) (Zhang *et al*., 2015).

Moreover, efficiency of the breeding programme will also depend on the genetic architecture of the traits which are under improvement (Cockerham, 1961). The understanding of gene action helps in the formulation of an efficient breeding programme since it provides exhaustive information about various gene interactions such as additive, dominance and non-allelic interactions. Overall, information like this is more authentic when attained over different environments and forms the foundation of any of the breeding programme. The present research was therefore undertaken on eight diverse germplasm lines to generate information on the nature and the magnitude of the gene action and combining ability effects for early maturity, seed yield, fibre yield and their component traits across environments so that the best parents and cross combinations can be used as apotential genetic resources for future crop improvement programmes in linseed.

Material and Methods

The experiment was conducted under sub temperate conditions of Western Himalayas during three successive *rabi* seasons, from 2015–16 to 2017–18 at the Experimental Farm of the Department of Crop Improvement, CSK HPKV, Palampur at an altitude of 1290.80 meters amsl at 32°80' N latitude and 76°33' E longitude. The experimental material comprised of eight linseed (*Linum usitatissimum* L.) genotypes and 28 cross combinations (F_1's) (Table 1). The cross combinations were attempted as per the 8 × 8 diallel mating design excluding reciprocals during *rabi* 2015-16.

Experimental design, data recording and statistical analysis

Eight linseed genotypes and 28 cross combinations (F_1's) were grown in a randomized complete block design (RCBD) with three replications at same location for two consecutive years i.e., *rabi* 2016-17 and *rabi* 2017-18. Each genotype and cross combinations were raised in a single row of 1.5 m with plant to plant and row to row spacing of 10 cm and 30 cm, respectively. The data were recorded from five random competitive plants in each line across replications on plant height, technical height, capsules per plant, seeds per capsule, primary branches per plant, secondary branches per plant, 1000-seed weight, seed yield per plant, aerial biomass per plant, harvest index, straw yield per plant, retted-straw yield per plant and fibre yield per plant, while phenological traits i.e., days to 50% flowering and days to 75% maturity were recorded on plot basis. To test the significance of differences among different genotypes/breeding material used in the study, the data on mean values for the different traits were analyzed as per Panse and Sukhatme (1985):

$$Y_{ij} - \mu_1 + g_1 + r_j + e_{ij}$$

where, Y_{ij} = phenotypic observation of i^{th} genotype grown in j^{th} replication, μ = general population mean, g_1 = effect of i^{th} genotype, r_j = effect of j^{th} replication, and e_{ij} = error component of i^{th} genotype in j^{th} replication.

Diallel Cross Analysis

The replication wise mean data obtained from F_1 population of twenty eight cross combinations for each trait were subjected to combining ability analysis of Griffing's (1956) Method 2 Model I.

When the F-test revealed significant differences among the genotypes, combining ability analysis was followed. A linear mathematical model for an observation made on ij^{th} genotype is expressed as:

$$Y_{ij} - \mu + g_i + g_j + s_{ij} + \frac{1}{bc}\sum_{K}\sum_{L} e_{ij}KL$$

where,

Y_{ij} = phenotype of the hybrid between i^{th} and j^{th} parents in k^{th} block

μ = population mean,

g_i = GCA effect of the i^{th} parent,

g_j = GCA effect of the j^{th} parent,

s_{ij} = SCA effect of the hybrid between i^{th} and j^{th} parents such that $s_{ij} = s_{ji}$

bc = block effect,

$e_{ij}kL$ = environment effect linked with $ijkL^{th}$ observation

$\frac{1}{bc}\sum_{k}\sum_{L} e_{ij}kL$ = is the mean error effect

Table 1. Linseed genotypes and their parentage/source used in the study

Sl. No.	Genotypes	Parentage/Source	Type	Seed colour	Maturity	Year of release	Reaction to Major diseases	
							Rust	Powdery mildew
1.	Surbhi	LC-216 × LC-185	Seed type	Yellow	Medium	1994	Resistant	Susceptible
2.	Bhagsu	RL-50-3 × Surbhi	Seed type	Brown	Late	2008	Moderately resistant	Susceptible
3.	Nagarkot	New River × LC-216	Dual purpose	Light brown	Medium	1995	Resistant	Resistant
4.	T-397	T491 × T1193-2	Seed type	Brown	Early	1984	Highly susceptible	Highly susceptible
5.	Himani	DPL-20 × KLS-1	Seed type	Brown	Late	2008	Moderately resistant	Moderately resistant
6.	Baner	EC-21741 × LC-214	Seed type	Light brown	Late	2005	Resistant	Moderately resistant
7.	JRF-4	CRIJAF, Barrackpore*	Fibre type	Brown	Early	-	Moderately resistant	Moderately resistant
8.	JRF-1	CRIJAF, Barrackpore*	Fibre type	Brown	Early	-	Moderately resistant	Moderately resistant

*[CRIJAF: Central Research Institue for Jute and Allied Fibre]

Combining ability analysis for pooled over environments

The combining ability analysis for pooled over environments for experimental Method 2 Model I was done by using method of Singh (1973). The notations used were: p (number of parents), b (number of blocks), c (number of observations) and L (number of environments).

The model used was:

$$Xijk = \mu + gi + gj + gj + Sij + LK + (gL)\,ik + (gL)\,jk + (SL)\,ijk + H + \frac{1}{bc}\sum_{n}\sum_{sijkn.s} e$$

where, μ = population mean, gi (gi) = GCA effect of i^{th} (j^{th}) parent, Sij = SCA effect of the crosses between the i^{th} and j^{th} parents, LK = effect of k^{th} environment, (gL)ik or (gL)jk interaction between GCA effects of the i^{th} and j^{th} parents with k^{th} environment, (SL)ijK = the interaction between SCA effects of the ij^{th} cross and the k^{th} environment, and H = 0 for fixed effect model.

Results and Discussion

The analysis of the variance revealed significant differences among the genotypes for all traits (Table 2). Variance due to environments in pooled analysis also indicated significant differences for the all traits studied except seeds per capsule suggesting considerable variation present amongst all the environments. The genotype×environment (G×E) interaction was also significant for all the traits except plant height and technical height. This indicates that genotypes behaved in a different way as the environmental conditions got changed and the variation in the stability of their performance over the environments was mainly due to G×E interaction. Yadav *et al*. (2014) observed significant G × E interaction for primary branches per plant, secondary branches per plant, days to maturity, number of seeds per capsule, 1000-seed weight, harvest index and seed yield per plant. Significant G × E interaction for different traits has been reported by Alem and Dessalegn (2014) and Temesgen *et al*. (2014). Parents vs. hybrids also expressed significant differences for all the traits studied indicating the existence of substantial amount of genetic variability.

The analysis of variance forthe combining ability affirmed mean squares due to GCA and SCA were significant for all the traits studied in pooled over environments (Table 2). Significant variation due to GCA and SCA indicates the importance of additive as well as non-additive types of gene action for the expression of the traits under study. These results are similar to the findings of Abdel-Moneam (2014), Abd Al-Sadek (2015), Singh *et al* (2016) and Kumar *et al* (2017).

The significant differences due to GCA, SCA and environments indicate the presence of sufficient genetic variability in the material. Furthermore, mean squares due to GCA×environment interaction were significant for all the traits studied except plant height, capsules per plant, seeds per capsule, harvest index, straw yield per plant, retted-straw yield per plant and fibre yield per plant, whereas mean square due to SCA×environment interaction were significant for all the traits studied except plant height, technical height and aerial biomass per plant. Significant mean squares due to GCA×environment and SCA×environment interaction effects indicated that the performance of the cross combinations and parents were affected by the environment and with the change in the environment combining abilities for traits did not remain same. Therefore, there is further need to test cross combinations as well as parents across the environments

Table 2. Variance and combining ability analysis for different traits in pooled over environments in linseed

	Variance over environments						Variance for combining ability					
Source of variation / Traits	Replication	Genotypes	Environments	Genotypes × Environment (g × e)	Parents vs. Hybrids	Error	GCA	SCA	Environments	GCA × Environment	SCA × Environment	**Pooled error**
df	2	35	1	35	1	140	7	28	1	7	28	**140**
Days to 50% flowering	20.14	67.92*	1166.69*	20.90*	95.72*	1.28	74.41*	9.70*	583.34*	5.89*	7.24*	0.43
Days to 75% maturity	4.68	105.13*	3978.38*	12.38*	13.76*	2.32	142.94*	8.07*	1989.19*	3.59*	4.26*	0.78
Plant height	74.95	275.85*	481.27*	23.63	732.60*	19.39	308.14*	37.90*	240.64*	6.71	8.17	6.46
Technical height	2.34	137.01*	610.31*	13.28	79.53*	8.91	185.89*	10.62*	305.16*	6.67*	3.87	2.97
Primary branches per plant	5.16	23.30*	17.39*	4.11*	433.34*	0.84	9.56*	7.32*	8.69*	1.55*	1.33*	0.28
Secondary branches per plant	4.67	13.57*	34.08*	3.96*	176.18*	0.46	6.56*	4.01*	17.04*	1.17*	1.36*	0.15
Capsules per plant	145.58	1041.22*	5054.03*	163.31*	21714.86*	40.74	356.08*	344.82*	2527.01*	17.70	63.62*	13.58
Seeds per capsule	0.67	1.65*	0.71	0.67*	1.82*	0.26	0.83*	0.48*	0.36*	0.13	0.25*	0.09
1000-seed weight	0.22	3.36*	18.82*	0.83*	36.51*	0.21	1.48*	1.03*	9.41*	0.18*	0.30*	0.07
Aerial biomassper plant	2.34	8.49*	211.38*	1.57*	96.58*	1.04	3.39*	2.69*	105.70*	1.06*	0.39	0.35
Seed yield per plant	0.15	1.59*	7.16*	0.23*	32.88*	0.04	0.49*	0.54*	3.58*	0.10*	0.07*	0.01
Harvest index	2.01	120.69*	39.42*	23.29*	1790.42*	9.81	55.43*	36.43*	19.71*	3.44	8.85*	3.27
Straw yield per plant	0.31	197.95*	789.67*	45.81*	3144.37*	28.55	77.61*	63.07*	394.84*	14.96	15.35*	9.52
Retted-straw yield per plant	0.33	1.40*	10.05*	0.21*	18.00*	0.11	0.77*	0.39*	5.03*	0.04	0.08*	0.04
Fibre yield per plant	0.10	29.93*	98.28*	3.89*	388.22*	2.50	18.23*	7.91*	49.14*	1.04	1.36*	0.83

* Significance at $P \leq 0.05$

mainly for traits showing significant interaction with the environment. Further, non-significant GCA×environment interaction and significant SCA×environment interaction for capsules per plant, seeds per capsule, harvest index, straw yield per plant, retted-straw yield per plant and fibre yield per plant indicated that the non-additive effects were more influenced by environment than the additive effects controlling these traits.

The estimates of genetic components of variance along with related genetic parameters revealed that the magnitude of σ^2SCA was higher than σ^2GCA for days to 50% flowering, plant height, primary branches per plant, secondary branches per plant, capsules per plant, seeds per capsule, 1000-seed weight, aerial biomass per plant, seed yield per plant, harvest index, straw yield per plant, retted-straw yield per plant and fibre yield per plant in combined over environments. The estimates of σ^2GCA were higher than σ^2SCA for days to 75% maturity and technical height in all the environments. The preponderance of σ^2SCA indicated predominant role of non-additive gene action governing all the traits except days to 75% maturity and technical height. This was also confirmed by ratio of σ^2GCA: σ^2SCA which is less than the theoretical maximum of unity for all the characters except days to 75% maturity and technical height in pooled over environments. The preponderance of non-additive gene action for primary branches per plant, secondary branches per plant, plant height, capsules per plant, seeds per capsule, seed yield per plant, 1000-seed weight and harvest index is in agreement with the findings of Nirala *et al*. (2018) and Mahto *et al*. (2019).

Further, σ^2A with higher magnitude, was noticed for days to 50% flowering, days to 75% maturity, plant height and technical height which exhibited the involvement of additive gene action. However, for the remaining characters σ^2D was higher than those of σ^2A in pooled over environments, signifying predominance of non-additive gene action (Table 3). The predominance of non-additive gene action has also been stated by Bhateria *et al*. (2006); Kumar and Paul (2015) and Naik (2017). In case of days to 50% flowering and plant height, the σ^2SCA was higher than σ^2GCA but contrary to that σ^2A was higher than σ^2D. It can be so because statistically GCA variance is the additive part of variability, besides it involves additive × additive and higher order of epistatic interactions (Matzinger and Kempthorne, 1956).

Over dominance (> 1) observed for primary branches per plant, secondary branches per plant, capsules per plant, seeds per capsule, 1000-seed weight, aerial biomassper plant, seed yield per plant, harvest index, straw yield per plant, retted-straw yield per plant and fibre yield per plant indicates the non-additive genetic variance in controlling the characters except for days to 50% flowering, days to 75% maturity, plant height and technical height where partial dominance was

Table 3. Estimation of genetic components of variance and degree of dominance for different traits in pooled over environments in linseed

Traits	σ^2_{GCA}	σ^2_{SCA}	$\sigma^2_{GCA}/\sigma^2_{SCA}$	σ^2A	σ^2D	Öσ^2D /σ^2A	Heritability (%) (h^2_{ns})
Days to 50% flowering	3.70	4.64	0.80	7.40	4.64	0.79	31.50
Days to 75% maturity	7.11	3.65	1.95	14.22	3.65	0.51	7.78
Plant height	15.08	15.72	0.96	30.17	15.72	0.72	28.77
Technical height	9.15	3.82	2.39	18.29	3.82	0.46	35.44
Primary branches per plant	0.46	3.52	0.13	0.93	3.52	1.95	11.14
Secondary branches per plant	0.32	1.93	0.17	0.64	1.93	1.74	13.27
Capsules per plant	17.12	165.62	0.10	34.25	165.62	2.20	9.15
Seeds per capsule	0.04	0.20	0.19	0.08	0.20	1.63	10.32
1000-seed weight	0.07	0.48	0.15	0.14	0.48	1.85	11.15
Aerial biomassper plant	0.15	1.17	0.13	0.30	1.17	1.96	8.62
Seed yield per plant	0.02	0.26	0.09	0.04	0.26	2.35	8.66
Harvest index	2.61	16.58	0.16	5.22	16.58	1.78	11.15
Straw yield per plant	3.40	26.78	0.13	6.81	26.78	1.98	8.01
Retted-straw yield per plant	0.04	0.18	0.21	0.08	0.18	1.56	13.60
Fibre yield per plant	0.87	3.54	0.25	1.74	3.54	1.43	14.94

* Significance at $P \leq 0.05$

observed indicating the predominance of additive gene action (degree of dominance being < 1) implying that selection may be effective in improving these characters (Table 3). The results are in confirmation with the findings of Kumar *et al*. (2016) and Singh *et al*. (2016).

Narrow sense heritability predicts the importance of the additive portion of the genetic variance that can be transferred to the next generation. The perusal of data presented in Table 3 revealed that medium heritability was observed for days to 50% flowering and technical height whereas, rest of the traits studied showed low heritability. Medium heritability suggests that the traits may be improved by making selections among the recombinants obtained through segregating populations. On the other hand, low heritability implies non-fixable component of the variation. As genetic variation of all the traits is mainly affected by the non-additive gene effects with low and medium heritability, so direct selection for such traits will not be effective. Therefore, these cross combinations can be utilized in later generations for getting desirable segregants. These result are consistent with the findings of Naik (2017) who has reported that narrow sense heritability estimates were low for plant height, primary branches per plant, capsules per plant, seeds per capsule, 1000-seed weight and seed yield per plant. Similarly, low narrow sense heritability for seeds per capsule, 1000-seed weight and seed yield per plant were reported by Goral and Ejsmond (2008).

General combining ability effects contribute significantly for identification of the germplasm base for its inclusion in the hybridization program. Out of eight genotypes *viz*.,Baner, Nagarkot and Surbhi were good general combiners for seed yield and its related traits. For fibre yield, the genotypes *viz*., JRF-1, JRF-4 and Bhagsu were found as the best general combiners. For dual purpose trait, genotype Nagarkot was observed as the good general combiner in pooled over environments. For early maturity, T-397, JRF-1and JRF-4 were found as the good general combiners (Table 4). In the present study of general combining ability effects, significant values were observed for maximum number of traits over combined environments in four genotypes *viz*., Nagarkot, Surbhi, JRF-1 andJRF-4 on the basis of their magnitude of the GCA effect. It seems that the GCA rank for economic yield is linked to the GCA for the important component characters. It can be inferred that besides the economic yield, these genotypes also possessed significant desirable GCA values for its component characters and appeared to be worthy for exploitation in practical plant breeding for utilizing the fixable component of variation. Therefore, these parents can be used extensively in the hybridization followed by selection to hasten the pace of the genetic improvement of dual purpose trait, seed yield, fibre yield and their component characters.

The desirable SCA effects were not revealed by any of the cross combination for all studied traits. However, four cross combinationswere selected for economic traits in pooled over environments on the basis of higher and significant specific combining ability effects in relation to GCA effects and *per se* performance. Himani×JRF-4, T-397×JRF-4, Bhagsu×Nagarkotand Surbhi×Baner were the promising cross combinations with desirable SCA effects for seed yield. The promising cross combinations indicating desirable SCA effects for fibre yield were Surbhi× T-397, Nagarkot×Himani, Surbhi×Baner and Surbhi×Bhagsu. In case of dual purpose trait, single cross combination with fascinating SCA effects was Surbhi×Baner. The combinations which exhibited high SCA effects involved all types of parents possessing good, average as well as poor GCA effects. Bhateria *et al*. (2006) and Singh *et al*. (2009) reported similar kind of results that the cross combinations indicating desirable SCA effects for different characters involve good×good, good×poor and good×average general combiners. Furthermore, in case of seed yield, only one cross combination, Surbhi×Baner (good×good) indicated significant positive SCA effects along with parents possessing good general combining ability effects for the trait. Therefore, necessarily it is not that the cross combinations showing higher SCA effects must involve good general combiners like their parents as observed by Sood *et al*. (2011). This suggests that GCA had no bearing on the SCA effects of the cross combinations (Bhateria *et al*., 2006).

Conclusion

The results obtained in the present investigation suggested that dominance gene effects were predominant as compared to additive effects for most of the traits including seed yield and fibre yield. Under such genetic control in the expression of traits, heterosis breeding may be useful but chances of exploiting hybrid vigour through hybrid varieties in linseed due to its autogamous nature are bleak at present. Under such situations, diallel selective mating or biparental mating in early segregating

Table 4. Estimates of general combining ability effects of parents for various traits in pooled over environments in linseed

Traits: Parental lines:	Days to 50% flowering	Days to 75% maturity	Plant height	Technical height	Primary branches per plant	Secondary branches per plant	Capsules per plant	Seeds per capsule	1000-seed weight	Aerial biomass per plant	Seed yield per plant	Harvest index	Straw yield per plant	Retted-straw yield per plant	Fibre yield per plant
Surbhi	1.23*	0.63*	-6.62*	-5.00*	0.82*	0.83*	3.56*	-0.06	0.16*	-0.63*	0.09*	2.54*	-4.00*	-0.21*	-0.62*
Bhagsu	0.35*	1.05*	2.73*	-0.02	0.29*	0.38*	2.99*	0.19*	-0.16*	0.27*	0.01	-0.63	1.19	0.14*	0.67*
Nagarkot	2.53*	2.21*	2.67*	1.52*	0.52*	0.54*	6.39*	0.04	0.17*	0.09	0.14*	1.40*	0.31	0.06	0.44*
T-397	-2.03*	-1.39*	-3.94*	-3.21*	0.61*	0.17*	-0.54	-0.45*	0.41*	-0.60*	-0.18*	-0.52	-1.88*	-0.24*	-1.50*
Himani	0.63*	2.30*	-2.06*	-1.07*	-0.25*	-0.39*	-2.19*	0.01	-0.41*	0.21	0.02	-0.30	0.98	-0.10*	-0.76*
Baner	1.78*	2.78*	-0.43	0.57	-0.06	-0.24*	0.34	0.07	0.00	0.55*	0.24*	1.42*	2.03*	-0.14*	-0.41*
JRF-4	-1.98*	-3.65*	3.45*	3.72*	-0.85*	-0.43*	-4.44*	0.19*	0.12*	0.08	-0.09*	-1.45*	0.48	0.25*	1.05*
JRF-1	-2.52*	-3.92*	4.20*	3.49*	-1.08*	-0.86*	-6.10*	0.01	-0.29*	0.03	-0.21*	-2.46*	0.90	0.23*	1.13*
SE (gi) ±	0.14	0.18	0.53	0.36	0.11	0.08	0.78	0.06	0.06	0.12	0.02	0.38	0.65	0.04	0.19
SE (gi-gj) ±	0.21	0.28	0.80	0.55	0.17	0.12	1.17	0.09	0.08	0.19	0.03	0.57	0.98	0.06	0.29
CD (gi)	0.27	0.35	1.05	0.71	0.22	0.16	1.54	0.12	0.11	0.24	0.04	0.74	1.28	0.08	0.37
CD (gi-gj	0.41	0.55	1.59	1.08	0.33	0.24	2.30	0.18	0.17	0.37	0.07	1.13	1.93	0.12	0.57

generations followed by pedigree selection method might be appropriate approach toward genetic improvement of these traits for exploitation of both fixable and non-fixable gene effects.Based on GCA effect, three genotypes namely Baner, Nagarkot and Surbhi were identified good general combiner for seed yield whereas genotypes, JRF-1 and JRF-4 were good for early maturity and fibre yield. These genotypes are identified for different characters and their promising characters can be combined to develop high yielding varieties. On the basis of high SCA effect, four cross combinations *viz*., Himani×JRF-4, T-397×JRF-4, Bhagsu × Nagarkot and Surbhi×Baner were found best for seed yield while four other *viz*., Surbhi×T-397, Nagarkot×Himani, Surbhi×Baner and Surbhi×Bhagsu were found best for fibre yield in pooled over environments. These promising genotypes and cross combinations can be used in further hybridization and selection can be done for seed, fibre and dual purpose trait as per the need of linseed breeding programmes.

Acknowledgments

We gratefully acknowledge our gratitude to the CSK HPKV, Palampur, India for the support while undertaking this research.

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Indian J. Plant Genet. Resour. 35(2): 217–223 (2022)
DOI 10.5958/0976-1926.2022.00024.9

RESEARCH ARTICLE

Assessment of Genetic Divergence for Yield and Yield Related Traits in Chilli (*Capsicum annuum* L.) Germplasm

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(Received: 30 October, 2021; Revised: 20 May, 2022; Accepted: 27 June, 2022)

Evaluation of 35 Chilli genotypes in two seasons during *Kharif*, 2019 and *Rabi*, 2019-20 was taken up with an objective to estimate the genetic divergence for yield and yield attributing traits. In respect of per plant fruit yield , Only one genotype IC-347044 (0.82 kg) recorded significantly higher value than the best check LCA-625 (0.65 kg). The genetic divergence of genotypes was estimated using Mahalanobis's D^2 statistics. The genotypes were grouped into six different clusters, among which cluster I was the largest comprising of 16 genotypes. Results revealed that the character capsanthin content contributed maximum (48.91%) towards diversity followed by ascorbic acid (47.23%), capsaicin content (1.85 %), number of fruits per plant (1.18%), fruit weight (0.67%) and chlorophyll content (0.17%). The inter cluster D^2 values revealed that the highest inter cluster generalized distance (4626.16) was between cluster IV and cluster V and the lowest (619.86) was between cluster I and cluster III. The genotypes belonging to the cluster IV (IC-526448, EC-378632, IC-561622) and V (IC-394819, IC-570408, EC-378688, IC-528442) should be crossed to develop superior chilli hybrids with yield and its component traits through heterosis breeding.

Key Words: Chilli, *Capsicum annuum,* Clusters, Genotypes, Divergence

Introduction

Chilli (*Capsicum annuum* L.) also known as hot pepper is an important Solanaceous vegetable widely cultivated throughout the world. Chilli is considered as one of the most important commercial spice crops and is widely used as universal spice. Indian chilli occupied an area of 7.33 lakh hectares (18.11 lakh acres) with a production of 17.64 lakh tonnes and productivity of 2400 Kg per hectare (971 Kg per acre) (FAO, 2019-20). In 2018-19, around 4-5 lakh tonnes of chilli was exported to countries like China, Sri Lanka, Bangladesh, U.A.E, Malaysia, Vietnam and Thailand.

Different varieties are cultivated for varied uses like pickles, vegetable, spice and oleoresin. The green chilli fruits are rich source of ascorbic acid, phytonutrients, carotenoids and rutin which are of immense importance in pharmaceutical needs (Purseglove, 1977). It is mainly used for its pungency and pleasant flavour (Saisupriya *et al.,* 2020). The pungency in chilli is due to the presence of group of alkaloids called capsaicinoids. Due to its anti-bacterial, anti-carcinogenic, analgesic and anti-diabetic properties, capsaicin is in high demand in pharmaceutical industry (Pallerla *et al.,* 2021).

Chilli belongs to the genus *Capsicum* which possesses enormous wealth of genetic diversity. Extent of genetic diversity determines the success level of crop improvement programme. Maximum diversity can be noticed among different cultivars available in India and outside with respect to shape, size, yield, quality and other traits. Germplasm collection, evaluation and maintenance of the genetic diversity in capsicum are important to avoid genetic erosion.

Genetic divergence is important as the germplasm are of wide varied origins and are highly variable regarding their production potential. Divergence analysis generates valuable information on the nature and degree of genetic diversity. Therefore, a clear characterization of germplasms is the first step to facilitate successful

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breeding efforts. It is essential for the breeder to choose the right type of parents for purposeful hybridization in heterosis breeding (Farhad *et al.*, 2010; Khodadabi *et al.*, 2011; Yatung*et al*., 2014). The degree of genetic divergence can be quantified using Mahalanobis's D^2 statistic of multivariate analysis which is recognized as a powerful tool for assessing the relative contribution of different characters to the total divergence (Das and Gupta 1984; Natarajan *et al.* 1988; Shidhu*et al.* 1989; Golakia and Makne 1992; Kalyani*et al.* 2017). In this regard, D^2 statistics was used in the present study to assess the genetic divergence of 35 chilli genotypes.

Materials and Methods

An investigation was undertaken with chilli accessions as treatments at College of Horticulture, Rajendranagar, Sri Konda Laxman Telangana State Horticultural University, Hyderabad during *Kharif*, 2019 and at National Bureau of Plant Genetic Resources Regional Station, Rajendranagar, Hyderabad during *Rabi*, 2019-20 to study genetic divergence. The accession numbers of the respective genotypes with source are presented in Table 1. Thirty five genotypes were grown in randomized block design and each treatment was replicated thrice. Nursery of 35 chilli genotypes was raised in pro trays with 50 cells. Six weeks old healthy seedlings were transplanted in the main field after allotting entries randomly in each replication. Each germplasm line was grown in a plot of 1.8 m × 4.2 m. All the recommended package of practicesprovided by university was adopted for raising a healthy crop.

Observations were recorded on 5 randomly tagged plants of each genotype in each replication for the traits *viz.,* plant height (cm), plant spread (cm^2), number of primary branches $plant^{-1}$, days to first flowering, days to 50% flowering, days to first harvest, days to last harvest, fruit length(cm), fruit diameter(cm), number of fruits $plant^{-1}$, fruit weight (g), fruit yield $plant^{-1}$ (kg/plant), fruit yield $plot^{-1}$(kg), ascorbic acid content (mg/100g of fruit), chlorophyll content of green chilli, capsaicin content (%) and capsanthin content (ASTA units). The capsaicin content (%) was estimated by procedure proposed by Palacio (1977). Capsanthin was estimated as per the procedure given by Ranganna (1986).

Pooled data from the two seasons (*Kharif*, 2019 and *Rabi*, 2019-20) was obtained and the genetic divergence between genotypes was estimated using Mahalanobis's D^2 statistics (1936). Since all the seventeen variables were correlated, they were transformed into uncorrelated linear combinations through pivotal condensation method. Procedure suggested by Tocher (Rao, 1952) has been used to group 35 genotypes into clusters by treating estimated D^2 values as the square of the generalized distance. The intra and inter-cluster distance were computed.

Table 1. List of chilli genotypes used for evaluation along with their sources

Tr. No	Genotype	Source	Tr. No	Genotype	Source
T_1	IC-347044	NBPGR Regional Station, Hyderabad	T_{19}	IC-528442	NBPGR Regional Station, Hyderabad
T_2	IC-363918	NBPGR Regional Station, Hyderabad	T_{20}	EC-399535	NBPGR Regional Station, Hyderabad
T_3	IC-363993	NBPGR Regional Station, Hyderabad	T_{21}	EC-378632	NBPGR Regional Station, Hyderabad
T_4	IC-561676	NBPGR Regional Station, Hyderabad	T_{22}	IC-215012	NBPGR Regional Station, Hyderabad
T_5	IC-561622	NBPGR Regional Station, Hyderabad	T_{23}	EC-378688	NBPGR Regional Station, Hyderabad
T_6	IC-610381	NBPGR Regional Station, Hyderabad	T_{24}	IC-214966	NBPGR Regional Station, Hyderabad
T_7	IC-505237	NBPGR Regional Station, Hyderabad	T_{25}	IC-319335	NBPGR Regional Station, Hyderabad
T_8	IC-447018	NBPGR Regional Station, Hyderabad	T_{26}	IC-394819	NBPGR Regional Station, Hyderabad
T_9	IC-572459	NBPGR Regional Station, Hyderabad	T_{27}	IC-572498	NBPGR Regional Station, Hyderabad
T_{10}	IC-610383	NBPGR Regional Station, Hyderabad	T_{28}	EC-399581	NBPGR Regional Station, Hyderabad
T_{11}	IC-214965	NBPGR Regional Station, Hyderabad	T_{29}	IC-526737	NBPGR Regional Station, Hyderabad
T_{12}	EC-402113	NBPGR Regional Station, Hyderabad	T_{30}	IC-570408	NBPGR Regional Station, Hyderabad
T_{13}	IC-410423	NBPGR Regional Station, Hyderabad	T_{31}	IC-561648	NBPGR Regional Station, Hyderabad
T_{14}	IC-526448	NBPGR Regional Station, Hyderabad	T_{32}	IC-334383	NBPGR Regional Station, Hyderabad
T_{15}	EC-399567	NBPGR Regional Station, Hyderabad	T_{33}	SINDHUR[c]	RARS , Lam , Guntur, AP
T_{16}	IC-561655	NBPGR Regional Station, Hyderabad	T_{34}	LCA-625[c]	RARS , Lam , Guntur, AP
T_{17}	EC-390030	NBPGR Regional Station, Hyderabad	T_{35}	PUSA JWALA[c]	IARI, New Delhi
T_{18}	IC-528433	NBPGR Regional Station, Hyderabad			

Results and Discussions

The mean performance of 35 genotypes of chilli in respect to 17 yield and yield contributing characters were studied. The mean performance of fruit yield per plant, capsanthin and capsaicin are only presented. Fruit yield per plant ranged from 0.11 kg to 0.82 kg with a total mean of 0.28 kg (Table 1). Among the genotypes, IC-347044 showed maximum fruit yield per plant (0.82 kg), while the minimum fruit yield per plant (0.11 kg) was observed in EC-390030 and IC-572498. Only one genotype IC-347044 (0.82 kg) recorded significantly higher value for fruit yield per plant than the best check LCA-625 (0.65 kg). The mean values for the quality trait capsaicin content ranged from 0.21 % to 0.83 % with a total mean of 0.44 %. Among the genotypes, maximum capsaicin content (0.83 %) was observed in IC-363993 followed by IC-570408 (0.80 %) which was at par, while EC-399567 showed minimum capsaicin content (0.21 %). Eleven genotypes *viz.,* IC-363993 (0.83 %), IC-570408 (0.80 %), IC-363918 (0.74 %), IC-526448 (0.71 %), IC-319335 (0.70 %), IC-410423 (0.67 %), EC-399535 (0.59 %), IC-561622 (0.58 %), IC-505237 (0.51 %), EC-390030 (0.51 %) and IC-561648 (0.50 %) recorded significantly higher values for capsaicin content than the check LCA-625 (0.42%). Capsanthin content ranged from 137.55 ASTA units to 373.52 ASTA units with a grand mean of 240.08 ASTA units. Among the genotypes, IC-561622 showed maximum capsanthin content (373.52 ASTA units), while the minimum capsanthin content (137.55 ASTA units) was observed in IC-610381. Eight genotypes serially IC-561622 (373.52 ASTA units), IC-526448 (322.18 ASTA units), EC-390030 (319.60 ASTA units), EC-378632 (311.15 ASTA units), IC-610383 (286.18 ASTA units), IC-215012 (283.67 ASTA units), IC-363918 (285.33 ASTA units) and IC-410423 (279.05 ASTA units) recorded significantly higher values for capsanthin content than the check LCA-625 (275.77 ASTA units).

The pattern of distribution of 35 genotypes into various clusters is indicated in Supplementary Table 1. All the genotypes evaluated were grouped into six clusters. Cluster I was the largest group comprising of 16 genotypes, followed by cluster III with 7 genotypes, cluster II and cluster V with 4 genotypes each, cluster IV with 3 genotypes, whereas cluster VI was monotypic or solitary with only one genotype suggesting diverse origin of this genotype. Pandit and Adhikary (2014)

Table 2. Mean values of fruit yield per plant (Kg), Capsaicin (%) and Capsanthin (ASTA units) in 35 chilli genotypes

Sl. No.	Genotype	Fruit yield per plant (Kg)	Capsaicin (%)	Capsanthin (ASTA units)	Sl. No.	Genotype	Fruit yield per plant (Kg)	Capsaicin (%)	Capsanthin (ASTA units)
1.	IC-347044	0.82	0.38	218.37	20.	EC-399535	0.15	0.59	212.66
2.	IC-363918	0.42	0.74	283.53	21.	EC-378632	0.28	0.40	311.15
3.	IC-363993	0.13	0.83	212.48	22.	IC-215012	0.27	0.32	283.67
4.	IC-561676	0.14	0.35	255.80	23.	EC-378688	0.13	0.31	199.42
5.	IC-561622	0.40	0.58	373.52	24.	IC-214966	0.18	0.46	191.92
6.	IC-610381	0.32	0.24	137.55	25.	IC-319335	0.18	0.70	203.17
7.	IC-505237	0.39	0.51	274.80	26.	IC-394819	0.14	0.31	165.33
8.	IC-447018	0.30	0.32	252.91	27.	IC-572498	0.11	0.24	253.41
9.	IC-572459	0.19	0.44	207.03	28.	EC-399581	0.20	0.42	227.98
10.	IC-610383	0.23	0.37	286.18	29.	IC-526737	0.13	0.26	186.36
11.	IC-214965	0.47	0.27	225.21	30.	IC-570408	0.56	0.80	175.06
12.	EC-402113	0.30	0.32	266.82	31.	IC-561648	0.17	0.50	222.41
13.	IC-410423	0.28	0.67	279.05	32.	IC-334383	0.13	0.38	247.00
14.	IC-526448	0.20	0.71	322.18	33.	Sindhur	0.46	0.35	240.95
15.	EC-399567	0.27	0.21	232.80	34.	LCA-625	0.65	0.42	275.77
16.	IC-561655	0.14	0.39	243.93	35.	Pusa Jwala	0.31	0.41	243.82
17.	EC-390030	0.11	0.51	319.60	–	Mean	0.28	0.44	240.08
18.	IC-528433	0.17	0.38	230.05	–	C.D. (5%)	0.06	0.04	2.63
19.	IC-528442	0.39	0.31	140.88	–	S.E. (m)	0.02	0.01	0.94

and Janaki *et al.* (2015) suggested that monogenotypic clusters were more divergent from others.

In order to get benefit of transgressive segregation, the knowledge of genetic distance between parents is necessary (Khodadabi *et al.,* 2011). In this regard, the mean intra and inter cluster D^2 values among the various clusters were estimated and were presented in the Supplementary Table 2. Similar studies were conducted by Varalakshmi and Haribabu (1991); Dutonde *et al.* (2008); Priyanka *et al.* (2018) and Manoj *et al.* (2019). Statistical distance represents the extent of genetic diversity among clusters. The intra cluster distance varied from 0.00 (Cluster VI) to 462.07 (Cluster V). Cluster II displayed least intra cluster distance denoting the similarity of genotypes. While maximum intra cluster distance was recorded in cluster V indicating the presence of sufficient amount of diversity with genotypes of the cluster. Thus, there is scope for selection among the genotypes within the clusters. Similar results were also obtained by Farhad *et al.* (2008), Datta *et al.* (2013); Yatung *et al.* (2014); Pujar *et al.* (2017)

The inter cluster distance was minimum between cluster I and III indicating narrow genetic diversity, whereas maximum recorded between clusters IV and V indicating wider genetic diversity in these groups. Similarly Indira (1994), Roy and Sorma (1996), Mishra *et al.* (2004), Yatung *et al.* (2014), Hasan *et al.* (2015), Sharma *et al.* (2017) also reported the presence of a high genetic divergence among chilli genotypes in their respective experiments.

The nearest and distant clusters from each of the cluster based on D^2 values are presented in Supplementary Table 3. Selection of parents from these diverse clusters for hybridization would help in achieving novel recombinants. The more diverse the parents within its limits of fitness, the greater are the chances of heterotic effects and broad spectrum of variability in segregating generations (Arunachalum *et al.* 1981). Therefore, it is logical to attempt crosses between genotypes falling in different clusters based on inter-cluster distance. This is simply to maximize overall genetic diversity and potential for genetic gain in the progeny (Nielson *et al.,* 2014).

The cluster means for each of the 17 traits are presented in Supplementary Table 4. From the data it can be seen that considerable differences exist for all the traits studied and they could be utilized as indicators for selecting diverse parents for specific trait in hybridization program (Farhad *et al.,* 2008; Smitha and Basavaraja, 2013, Sharma *et al.* 2017). Cluster VI recorded highest mean plant height (84.83 cm) followed by cluster IV (68.59 cm). While, cluster I registered lowest mean plant height (55.15 cm). Plant spread was highest in cluster VI (6712.00 cm^2) followed by cluster II (5051.79 cm^2). While cluster III (3073.12 cm^2) recorded lowest plant spread. The character numbers of primary branches per plant were highest in cluster VI (5.13) followed by cluster IV (3.46), whereas less number of primary branches was observed in cluster III (3.11).

Cluster V recorded maximum number of days to first flowering (62.09) followed by cluster IV (61.88). While the cluster III recorded least number of days to first flowering (52.89). Days to fifty per cent flowering recorded minimum value in the cluster III (60.57 days). While the cluster IV (66.33 days) exhibited maximum mean value followed by cluster VI (65.33 days). The number of days for first harvest was observed to be minimum (83.76) among the genotypes of the cluster III, while the maximum number of days (98.00) has been observed among the genotypes of the cluster VI. The genotypes grouped into cluster I recorded lower number of days (147.73) to last harvest, while the genotypes of the cluster VI showed highest number of days (162.33) for last harvest. Cluster V (9.82 cm)

Table 3. Percent contribution of different characters towards genetic divergence in thirty five chilli genotypes

S No.	Character	Rank	Contribution
1	Plant height (cm)	0	0%
2	Plant spread (cm^2)	0	0%
3	No. of primary branches per plant	0	0%
4	Days to first flowering	0	0%
5	Days to 50 % flowering	0	0%
6	Days to first harvest	0	0%
7	Days to last harvest	0	0%
8	Fruit length (cm)	0	0%
9	Fruit diameter (cm)	0	0%
10	No. of fruits/plant	7	1.18%
11	Fruit weight (g)	4	0.67%
12	Fruit yield per plant (Kg)	0	0%
13	Fruit yield per plot (Kg)	0	0%
14	Ascorbic acid (mg/100g)	281	47.23%
15	Chlorophyll content (%)	1	0.17%
16	Capsaicin content (%)	11	1.85%
17	Capsanthin content (ASTA unis)	291	48.91%

recorded highest fruit length followed by the cluster II (9.22 cm). Whereas, the cluster VI (2.77 cm) recorded lowest fruit length. Highest fruit diameter was recorded in the cluster VI (2.06 cm) followed by the cluster III (1.33 cm) whereas, lowest fruit diameter was recorded in the cluster IV (1.01 cm).

The mean values for maximum number of fruits per plant were recorded by the cluster IV (94.33) followed by cluster II (81.33), while minimum number of fruits per plant was recorded by the cluster VI (55.33). Maximum fruit weight was observed in the cluster VI (6.08 g) followed by the cluster V (5.85 g). While the cluster IV (3.52 g) recorded minimum fruit weight. The character fruit yield per plant was recorded maximum in the cluster II (0.35 kg) followed by the cluster V (0.34 kg). While the cluster I (0.27 kg) recorded minimum fruit yield per plant. The character fruit yield per plot was recorded maximum in the cluster II (4.95 kg) followed by the cluster V (4.80 kg). While the cluster I (3.80 kg) recorded minimum fruit yield per plot.

Ascorbic acid content of the fruits was found to be highest (182.54) in cluster II followed by cluster V (127.27), while the lowest ascorbic acid content (46.24) was noted among the genotypes of the cluster VI. Chlorophyll content of the green fruits was found to be highest (2.08) in cluster VI followed by cluster I (1.95), while the least chlorophyll content (1.79) was observed among the genotypes of the cluster V. The genotypes of the cluster IV showed highest capsaicin content (0.59) followed by (0.48) among the genotypes of the cluster I, while the genotypes of the cluster VI recorded lowest capsaicin (0.25). Capsanthin content was observed to be highest (336.25) in cluster IV followed by (269.80) among the genotypes of the cluster III, while the lowest capsanthin content (138.40) was recorded among the genotypes of the cluster VI.

Genetic divergence among thirty five genotypes revealed that the genotypes *viz*., IC-363918, IC-610381, EC-378632, IC-363993 and Pusa Jwala were identified as promising genotypes for plant height. Hence, these genotypes can be utilized in crop improvement programme as donor parents for improving plant height. The genotypes *viz.,* IC-215012, IC-610381, IC-570408 and Pusa Jwala are promising genotypes for improving plant spread. The genotypes IC-215012, IC-610381, IC-528433 and Pusa Jwala are promising for improving number of primary branches per plant. The genotypes IC-561655, IC-610383, IC-528433, IC-363993, IC-347044, Pusa Jwala, LCA-625 and IC-363918 were found to be promising lines for less number of days to first flowering and 50% flowering. Five genotypes *viz.,* IC-561655, IC-610383, LCA-625, Pusa Jwala and Sindhur for days to first harvest and another two genotypes, IC-447018 and IC-610383 for days to last harvest were found to be promising. The other promising genotypes include IC-347044, LCA-625, IC-570408, Pusa Jwala, EC-378688, IC-214965, IC-572459, EC-390030 and Sindhur for fruit length, IC-610381 and Sindhur for fruit diameter, IC-363918, IC-347044, LCA-625, Pusa Jwala and IC-410423 for number of fruits per plant, Sindhur, EC-378688, IC-561655, IC-214965, IC-570408, IC-572459 and IC-347044 for fruit weight. IC-347044, LCA-625 and IC-570408 can be potentially used for improving the yield.

Six genotypes *i.e.,* IC-215012, Pusa Jwala, IC-214965, EC-399581 and IC-528442 for ascorbic acid, EC-402113, EC-399535, IC-610383, IC-561655 and IC-215012 for chlorophyll content, IC-363993, IC-570408, IC-363918, IC-526448 and IC-319335 for capsaicin content, IC-561622, IC-526448, EC-378632 and EC-390030 for capsanthin content can be potentially used for improving the respective characters.

The percent contribution of different characters towards genetic divergence was presented in Table 3. The relative contribution of capsanthin content was maximum (48.91 %) towards diversity by taking 291 times ranking first followed by ascorbic acid (47.23 %) by 281 times, capsaicin content (1.85 %) by 11 times, no. of fruits per plant (1.18 %) by 7 times, fruit weight (0.67 %) by 4 times and chlorophyll content (0.17 %) by 1 time. Similar results were reported by Srinivas *et al.* (2021). In contrast, plant height, plant spread, number of primary branches per plant, days to first flowering, days to 50% flowering, days to first harvest, days to last harvest, fruit length, fruit diameter, fruit yield per plant and fruit yield per plot did not contribute towards total diversity.

Conclusion

The present study revealed that considerable genetic diversity exists among and within the six clusters. Selection of parents from these diverse clusters for hybridization programme would help in achieving novel recombinants. Hence, apart from selecting genotypes from the clusters which have high inter-cluster distance for hybridization, one can also think of selecting parents

based on extent of genetic divergence in respect to a particular character of interest. This means, if breeder's intention is to improve fruit yield, he can select parents which are highly divergent with respect to that character. Emphasis should be laid on characters contributing maximum D^2 values for choosing the cluster for the purpose of further selection and choice of parents for hybridization. The novel genotypes identified in this study could be utilized in further genetic studies.

Acknowledgements

The authors are highly thankful to SKLTSHU, Mulugu, Siddipet and NBPGR, Rajendranagar, Telangana for providing all facilities to complete this endeavour.

Conflict of Interest

No conficts of interest exist among the authors.

*Supplementary Table or Figure mentioned in the article are available in the online version.

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Supplementary Table 1. Clustering pattern of thirty five chilli genotypes (Tocher's method)

Clusters	Genotypes
I	IC-528433, IC-561648, EC-399535, IC-363993, IC-319335, IC-214966, IC-526737, IC-347044, IC-334383, IC-447018, IC-561676, EC-402113, IC-410423, IC-572498, IC-572459, LCA-625
II	IC-214965, EC-399581, PUSA JWALA, IC-215012
III	IC-505237, IC-610383, EC-390030, IC-561655, SINDHUR, EC-399567, IC-363918
IV	IC-526448, EC-378632, IC-561622
V	IC-394819, IC-570408, EC-378688, IC-528442
VI	IC-610381

Supplementary Table 2. Average intra (bold) and inter-cluster D^2 values for six clusters in thirty five genotypes of chilli

Cluster	I	II	III	IV	V	VI
I	**316.38**	2289.29	619.86	1420.32	1519.31	946.28
II		**206.24**	1277.10	4387.88	1067.69	3282.76
III			**325.24**	1383.09	1493.98	1930.75
IV				**269.66**	4626.16	3776.11
V					**462.07**	1192.37
VI						0.00

Supplementary Table 3. The nearest and farthest clusters from each cluster based on D^2 values in chilli genotypes

Cluster number	Nearest cluster with D^2 values	Farthest cluster with D^2 values
I	**III** (619.86)	**II** (2289.29)
II	**V** (1067.69)	**IV** (4387.88)
III	**I** (619.86)	**VI** (1930.75)
IV	**I** (1420.32)	**V** (4626.16)
V	**II** (1067.69)	**IV** (4626.16)
VI	**I** (946.28)	**IV** (3776.11)

Supplementary Table 4. Mean values of clusters for 17 characters in 35 chilli genotypes (Tocher's method)

Clusters	Plant height (cm)	Plant spread (cm^2)	No. of primary branches per plant	Days to first flowering	Days to 50% flowering	Days to first harvest	Days to last harvest	Fruit length (cm)	Fruit Diameter (cm)	No. of fruits per plant	Fruit weight (g)	Fruit yield per plant (Kg)	Fruit yield Per plot (Kg)	Ascorbic acid(mg/100g)	Chlorophyll (%)	Capsaicin (%)	Capsanthin (ASTA units)
I	55.15	3494.92	3.38	57.59	62.35	91.19	147.73	8.03	1.08	70.23	3.99	0.27	3.80	67.26	1.95	0.48	233.25
II	59.68	5051.79	3.30	56.31	62.00	89.50	158.92	9.22	1.12	81.33	4.83	0.35	4.95	182.54	1.81	0.38	246.97
III	59.93	3073.12	3.11	52.89	60.57	83.76	152.05	7.42	1.33	68.95	5.76	0.32	4.45	109.03	1.93	0.46	269.80
IV	68.59	4491.33	3.46	61.88	66.33	91.22	151.00	8.57	1.01	94.33	3.52	0.33	4.62	53.53	1.87	0.59	336.25
V	56.73	3897.15	3.24	62.09	63.92	93.67	153.92	9.82	1.12	67.42	5.85	0.34	4.80	127.27	1.79	0.47	168.77
VI	84.83	6712.00	5.13	56.00	65.33	98.00	162.33	2.77	2.06	55.33	6.08	0.33	4.67	46.24	2.08	0.25	138.40

Indian J. Plant Genet. Resour. 35(2): 224–232 (2022)
DOI 10.5958/0976-1926.2022.00025.0

RESEARCH ARTICLE

Elevated Temperature Disrupts Pollen-Pistil Dynamics and Seed Set in Okra (*Abelmoschus esculentus* L. Moench)

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(Received: 17 December, 2021; Revised: 27 March, 2022; Accepted: 04 April, 2022)

Elevated temperature can interfere with pollen formation and function in okra (*Abelmoschus esculentus*). The study was aim to quantify the impact of elevated temperatures on the reproductive stage in okra. In both the stain analysis and pollen tube growth test, pollen viability was decreased at elevated temperatures. The highest number of non-viable pollen grains were observed at 35°C and 40°C. The stigma was nonsignificantly high in receptivity at all temperatures; however, the seed set showed a significant decline under elevated temperatures. The findings offer the potential to look further into approaches, to genetic enhancement of heat-tolerant plants that will secure *okra* productivity during future climatic variation.

Key Words: Fertility, Fruit formation, Heat stress, Pollen, Stigma receptivity

Introduction

The rise in temperature due to global warming is a concern in many parts of the world (Anderson *et al.*, 2017; Feng *et al.*, 2017; Lobell and Asseng 2017). A report said, a 2°C increase would greatly exacerbate extreme weather, rising sea levels, loss of ecosystems, arctic melting, and other impacts (Anonymous, 2018). Even if governments were to implement their pledges fully, the world would face a rise in mean temperatures of 2.4 to 3.8°C by 2100. Elevated temperature caused an adverse impact during specific development of pre-zygotic and post-zygotic stages in okra (*Abelmoschus esculentus* L. Moench) (Ganpat and Isaac, 2015; Müller *et al*., 2016 and Broussard *et al*., 2017). Sexual reproduction of okra is sensitive to elevated temperature with reproductive tolerance up to 30 to 32°C (Arulrajah and Ormrod, 1973; Mangrich and Saltveit, 2000; Rahman *et al.*, 2012). Increased temperature caused adverse effects on seed set, which results in reduced seed dormancy and final seed yield due to alteration in floral development (Hoque *et al.*, 2016; Balasubramanian *et al.*, 2006; Oloumi and Rezanejhad, 2009). Responses may differ significantly between ecotypes of the same species (Madan *et al.*, 2012; Huang *et al.*, 2014). While temperature sensitivity has been extensively studied using leaves and roots (Iba, 2002; Yamaguchi-Shinozaki and Shinozaki 2006, Kotak *et al*., 2007; Wahid *et al*., 2007; Aubry-Kientz *et al*., 2019). Studies on sexual reproduction are more complicated because gamete development and fertilization are complex processes occurring during a short period, and predominantly hidden within the flower. Pollen-pistil dynamics under elevated temperature in okra requires elucidation. The study was undertaken to determine the influences of elevated temperature on pollen-pistil dynamics and seed formation in okra.

Material and Methods

Okra seed, cv. Clemson Spineless (CS), was obtained from the National Agriculture Research and Extension Institute (NAREI), Guyana, South America. On the Sixteenth day of September seeds pretreated with pesticides were sown into the potting mix soil in the germination tray. Water was provided immediately after sowing and then every day in the morning and afternoon until plants were 21 days old. While seedlings were developing seedbeds under the shade house were measured and arranged in a completely randomized design (CRD) with 3 replications of 4 treatments on 12 plots, the beds are denoted as plots. Soil preparation of the plots included the incorporation of poultry manure where 25 kg of manure per plot were added and mixed with the soil, then allowed to rest for 5 to 7 days before planting. All recommended agronomic practices were

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followed to raise the right crop. Soon the plots were ready for planting.

Developed seedlings in the germination tray were transplanted onto the prepared CRD plots inside the shade house on the sixth day of October. In each plot, seedlings were planted in double rows with 6 plants in a row, 12 plants per plot, with a spacing of 88 cm within the row and 90 cm between rows (Olczyk *et al*., 2005, 2006). Plots were $5m^2$ (5.0×1.0 m). Plants were watered immediately after transplanting and then continuously watered every morning and afternoon until plants matured and research was completed.

Pollen viability was determined with the Carmine Acetic Acid (CAA) stain using mature anthers (Sheidai and Fadaei, 2005). Carmine acetic acid stain was prepared by boiling a 40% acetic acid solution saturated with carmine. Flowers were harvested during morning hours and incubated for 2 h at 25 (control), 30, 35, or 40°C. Pollen grains were dusted onto a slide containing 1-2 drops of CAA stain, allowing immersion of pollen in the stain for 20-30 min. Viability was determined by counting darker stained pollen (viable), non-stained, or lightly stained and ruptured pollen (non-viable), using an OPTIKA Compound Light microscope (Ponteranica, Italy) at 400× magnification.

Each morning, freshly opened flowers and buds one day after anthesis were harvested and incubated at each temperature treatment for 2 h. Placing un-dehisced anthers in incubators allowed anthers to dehisce exposing pollen grains. Temperature treated pollen was cultured onto basic pollen germinating medium (PGM) for 1 h. A slightly modified *in-vitro* pollen growth medium (Li *et al*., 1999) was prepared which contained 0.01% boric acid; 5 mM calcium chloride; 5 mM potassium chloride; and 1 mM magnesium sulfate. The pH 7.5 was maintained with 1M potassium hydroxide and 20% sucrose for the solid medium as a source of carbohydrate and 1.5% agarose for solidification of media. Pollen germination was observed with the OPTIKA Compound Light microscope. Pollen grains were considered to have germinated, or be viable when the pollen tube had gained a length equal to, or longer than, the diameter of the pollen grain. Bursting pollen grains were categorized by an irregular mass of cytoplasm and starch grains protruding from the cells (Adhikari and Campbell, 1998; Kakani *et al*., 2002).

Stigma receptivity was determined in 40 flowers from all replicates where flowers were emasculated 1 day before anthesis. Immediately after emasculation, the flowers were placed within paper bags to prevent unwanted pollination. Receptivity of stigma was studied the next day (day 2). Emasculated flowers were harvested during morning hours and placed in an incubator for 2 h at 25, 30, 35, or 40°C. After removal from the incubator; the stigma surface was cut with a sharp razor blade, and 6% hydrogen peroxide solution (H_2O_2) was applied on the cut (decapitated) surface with a dropper. The appearance of bubbles within 2 to 3 min on the stigma surface indicated it was receptive, according to the methodology proposed by Silva *et al.*, 2013 and Gupta *et al*., 2015.

Approximately 20 mature unopened flowers per replicate of all treatments were hand-emasculated a day before hand-pollination. Emasculated flowers were covered in paper envelopes to prevent unwanted pollination. The next morning between 8.00 and 10.30 am, open flowers and dehisced anthers were collected and brought to the laboratory; where the flowers with exposed pollen grains were treated at 25, 30, 35, and 40°C for 2 hours. On the same day, the flowers were artificially pollinated by dusting with temperature-treated pollen, collected from incubated flowers, directly onto the stigma surface of the emasculated flowers. Pollinated flowers were bagged, and set aside to determine if fertilization occurred. Pistils were left on plants until maturity to determine seed sets with continuous monitoring. On day 12 following pollination, mature fruit was collected, and the seed set was counted. Statistical analysis was conducted using Analysis of Variance (ANOVA) in Statistics 10.

Results and Discussion

Pollen viability and germination

To test whether high-temperature influences pollen viability in *A. esculentus*, temperature-treated pollens were stained with carmine acetic acid. Consequently, a higher number of lightly stained, unstained, and ruptured pollen was noted for higher temperature treated pollen. In contrast, the proportion of darkly stained pollen was noted higher in number at ambient temperature treatment of 25°C. Hence overall pollen viability was recorded, substantially lower for increased temperatures, with a mean percentage of 70.1% at 40°C with 74.98% at 35°C and 79.8% at 30°C, respectively (Fig. 1a). On the contrary, at an ambient temperature of 25°C mean percentage was 82.0%, respectively. This suggested

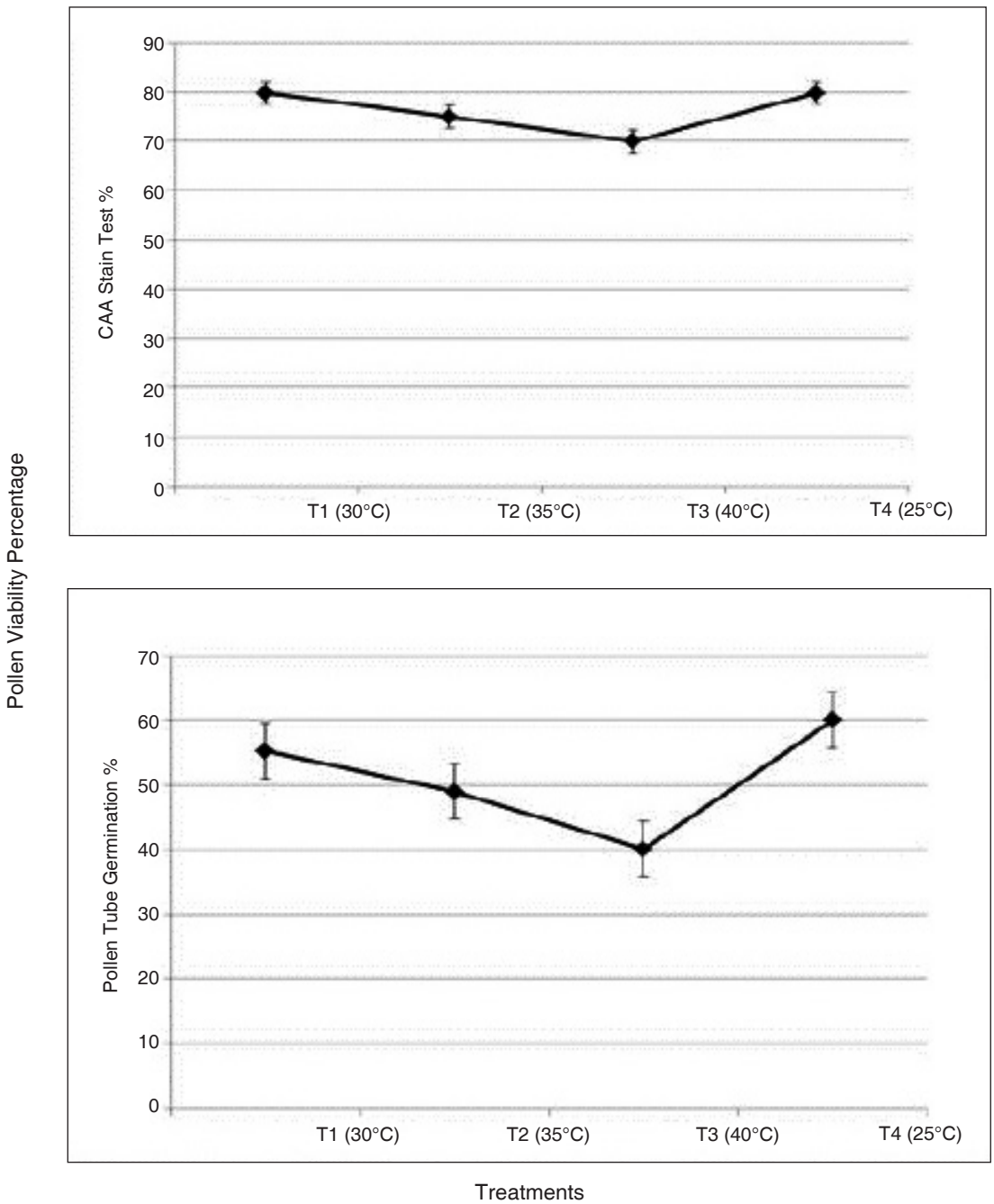

Fig. 1. Temperature effect on pollen viability: pollens in CAA stain (a) and pollen growth medium PGM (b)

that there was a significant effect of high temperature ($P < 0.05$) on pollen viability. The highest temperature (40°C) showed the least viable pollen grains, while the control (25°C) had the highest count of viable pollens. The highest pollen germination rates for the *A. esculentus* were detected (i.e. 60.02%) at an optimal temperature of 25°C (control). The germinability decreased to 55.28% at 30°C, 49.02% at 35 ºC, and 40.1% at 40°C (Fig.1b). Darkly stained pollen is regarded as highly viable, while the lightly stained or unstained, or ruptured were considered non-viable (Fig. 2a-c). Pollen germination percentage was calculated as the proportion of pollen grains germinated to the total number of pollen grains observed (Fig. 3 a-b). Analysis of variance (ANOVA)

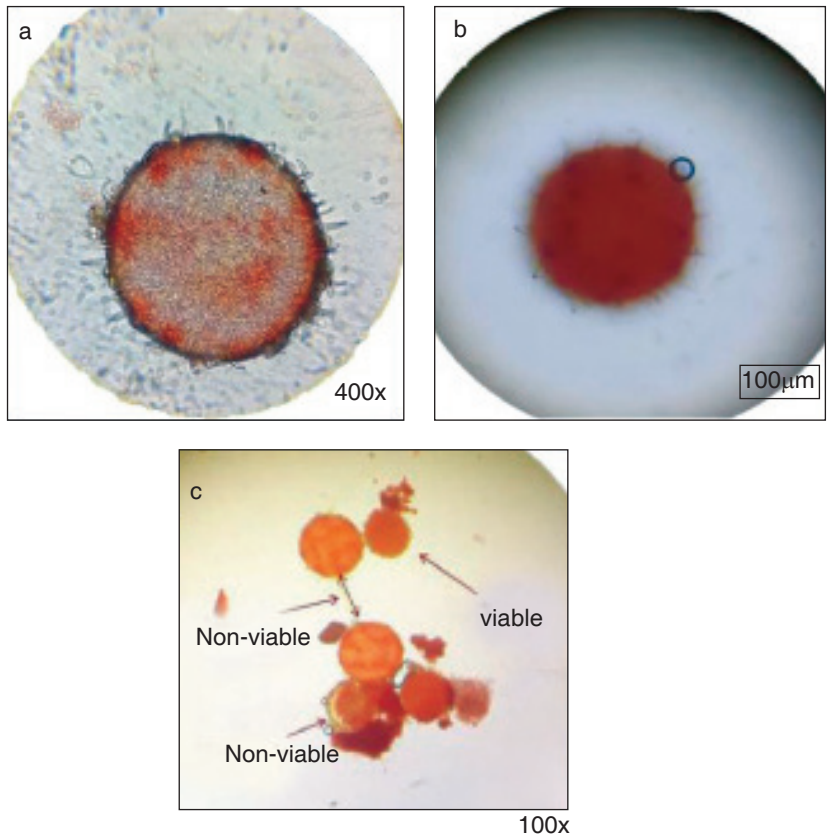

Fig. 2. Pollen viability of *A. esculentus* stained with CAA, non-viable (unstained) pollen (a), viable pollen (darkly stained) (b), and both viable and nonviable (C).

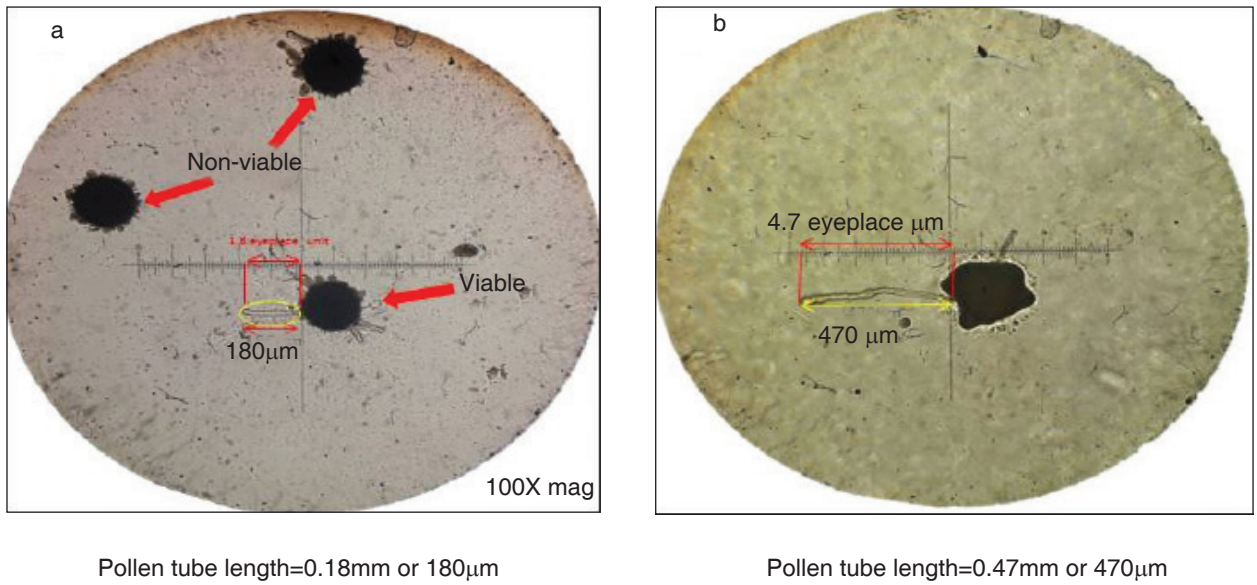

Fig. 3. *In-vitro* pollen tube elongation of viable and non-viable pollen of *A. esculentus* on pollen germination medium observed under the compound microscope (a) and (b)

showed that the higher temperatures had significant effects on pollen tube elongation, reducing pollen viability ($P < 0.05$). It is widely accepted that sexual reproduction in plants is highly vulnerable to temperature (Hedhly *et al.*, 2003; Hedhly *et al.*, 2009). The greatest sensitivity to an elevated temperature at early reproductive stages found in this study was declined pollen viability in stain test and *in-vitro* pollen germination of okra with reduced seed set. As many studies had shown pollen viability in the number of crops such as tomatoes (Müller *et al*., 2016), Arabidopsis (Huang *et al*., 2014), rice (Liu *et al*., 2004), and peach (Herrero and Arbeloa, 1989) is reduced at elevated temperatures. Earlier studies on cotton pollen have shown that temperatures (>30°C) inhibit *in-vitro* pollen growth and pollen tube penetration into pistil structures (Barrow, 1983; Kakani *et al.*, 2005).

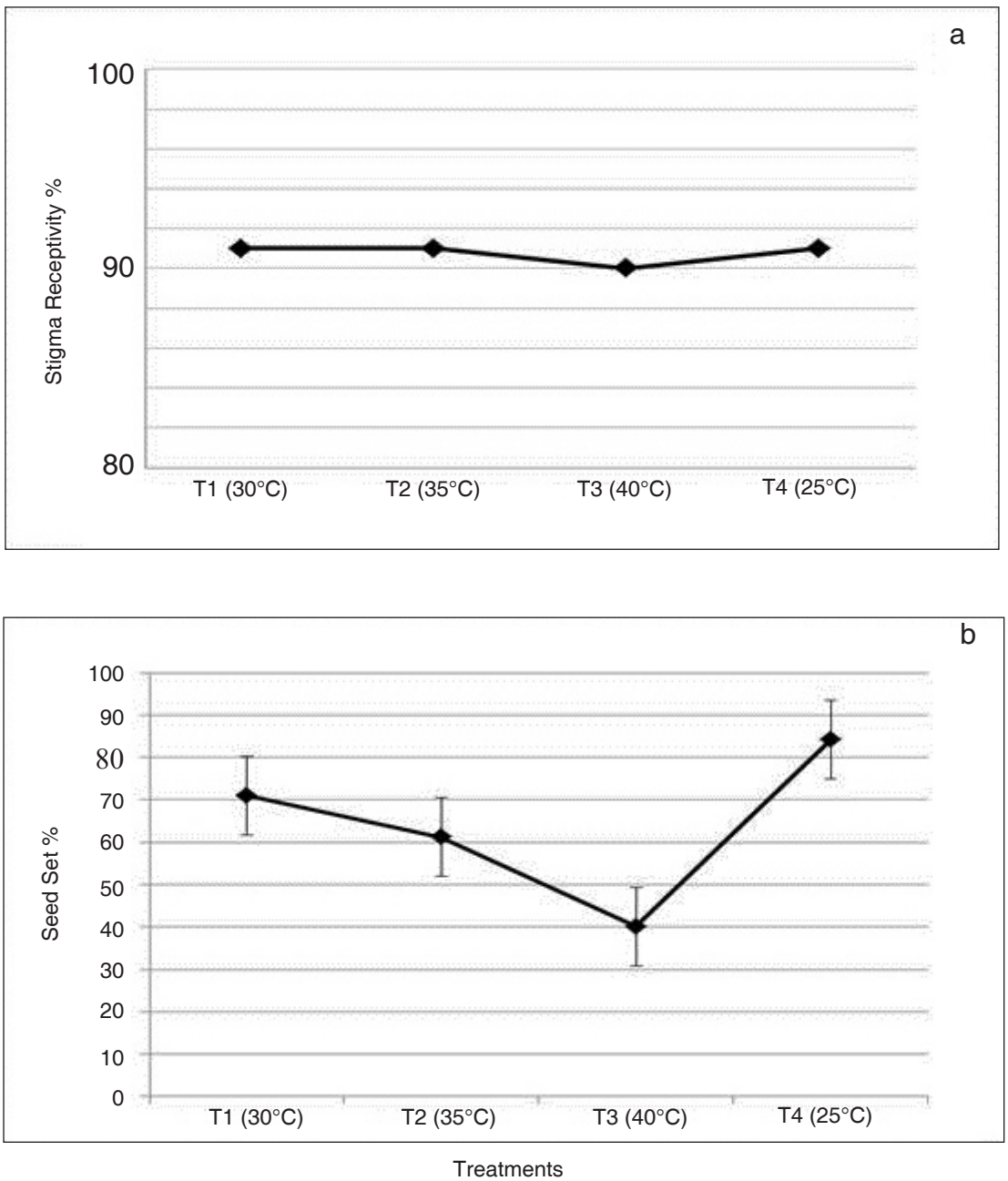

Fig. 4. Stigma receptivity (a) and seed set (b) under different temperature treatments. For (a) the p-value = 0.8018; with no significant difference in stigma receptivity with an increase in temperature. For (b) the p<0.05 with a significant difference in seed set with an increase in temperature.

In the current study, a significant percentage of pollen viability was observed amongst varied temperatures 35 to 40°C showed reduced viable pollens with deteriorated microspore cytoplasmic contents, which appeared as lightly stained or unstained. The percentage for non-viability in pollen was recorded, 70.1, 74.9, and 79.8% at high-temperature, 40, 35, and 30°C respectively, compared to control that showed viability percentages of 82.01% with darker stained pollens. *In-vitro* pollen germination n showed reduced pollen viability percentage, 55.21, 49.0, and 40.1% at temperatures 30, 35 to 40°C respectively. That contributes to a much lower proportion of pollen tube elongation; considerably tube length had not exceeded the substantial diameter of the pollen. On the contrary, it was found that dynamic tube elongation at 25°C of temperature having 60.02%. Therefore, the higher temperatures had a significantly negative impact on the feasibility of male gamete and consequently hindered the reproductive processes in plants reducing yield.

Stigma receptivity

The analysis of variance (ANOVA) showed that various temperatures of, 25, 30, 3,5 and 40°C respectively had no significant effect on stigma receptivity (P>0.05) (Fig. 4a). Generally, all-temperature treated samples had a similar trend, i.e., 91, 91, 91, and 90% for 25, 30, 35, and 40°C, respectively. In all the analyzed flowers, stigma was fully receptive. Oxygen bubble formation within 1-3 minutes observed on the stigmas was considered receptive (Fig. 5a). Elevated temperatures had no significant effect on stigma receptivity. All different temperatures *i.e.,* 25, 30, 35, and 40°C recorded a receptivity percentage of stigma ranging between 90–91%. For instance, pollen has been reported to be more sensitive to higher temperatures than female reproductive structures (Balasubramanian *et. al*., 2006). However, the effects of high temperature on female fertility could not be disregarded (Mangrich and Saltveit, 2000; Porch and Jahn, 2001). The previous report revealed that exposure to a high-temperature response in snap bean did not significantly affect stigma receptivity (Dickson and Boettger, 1984). Studies with Indian mustard (*B. juncea*) suggested vulnerability in stigma receptivity when exposed to high temperature during at flowering stage (Maity *et al*., 2019).

Seed Set

The temperature had a more significant effect on the seed-set; with the lowest seed set of 40.1% that was obtained under an elevated temperature of 40°C followed by a 5°C temperature treat with mean having 61.23% seed set (Fig. 4b). Seed-set was reduced by increased temperatures with a significant relationship between seed set and elevated temperature ($P < 0.05$) (Fig. 5b-c). It was evident that the highest seed set (84.21 %) was found under ambient temperature (25°C) treatment followed by treatments under 30°C of temperature (70.9%). Likewise, higher temperatures also instigated bell shape fruit formation (Fig. 5d). The result indicated that the elevated temperature decreased seed set count and allowed the deformation of fruits. There was an obvious negative relationship obtained between temperature and seed set. The effects of temperature above the critical temperatures (35/40°C) had recorded a reduced seed set supported by the previous studies showed a reduction in pollen viability and seed set in beans (Monterroso and Wien, 1990; Gross and Kigel, 1994). In our study, there was no effect on the fertilization process and seed set at 25°C; however, the seed set number decreased as temperature increased above 30, 35 to 40°C. Interestingly, fruits produced at temperatures 35 and 40°C were noticed to have taken bell-shaped and did not have fully developed seeds. Thus, it is determined that after exposure to temperatures 30, 35, and 40°C, respectively, there were fewer pollen grains per flower that remained viable. Consequently, studies suggested that reduced seed-set at higher temperatures is likely a result of lower anther dehiscence and pollen sterility (Monterroso and Wien, 1990; Gross and Kigel, 1994). Homogenous effects on pollen development and fruit-set have been observed in peanut (Prasad *et al*., 2002, 2003), cowpea (Hall, 2004), and tomato (Peet *et al*., 1998). Generally, plant response to high temperature was found to be most severe during periods of rapid growth and development (Hoque *et al*., 2016). Little tolerance of pollen development to heat stress has been reported in Chinese cabbage (Kuo *et al.*, 1981) and bottle gourd (Iapichino and Loy, 1987). Gibberellin regulates floral developments (Gupta and Chakrabarty, 2013) thus; an increase in temperature leads to GA-deficiency that caused flower mutation typically having short stamens as a result of reduced cell extension within the filaments the lowest pod set was observed in snap bean when flower buds were exposed to heat, which gradually decreased floral development affecting young pods and seed-set rate (Dickson and Boettger, 1984).

The elevated temperature adversely affects the male reproductive phase in *A. esculentus*, which is amongst

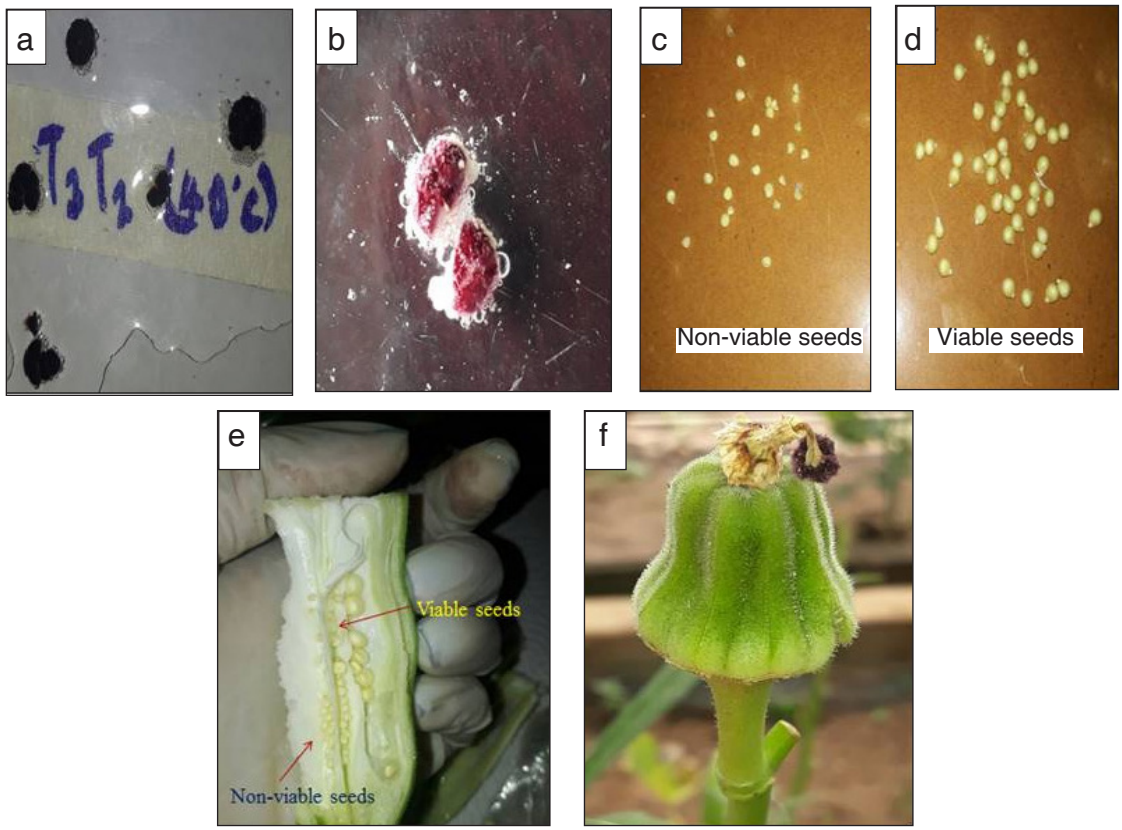

Fig. 5. Female reproductive structure (stigma) of *A. esculentus* with numerous oxygen bubbles on the stigma after reaction with hydrogen peroxide (a-b). Seeds not fully developed; reduced in size and irregular shaped (Non-viable seeds) (c), seeds fully developed; larger in size and plump shaped (Viable seeds) (d), viable and non-viable seeds attached to Okra fruit, obtained after artificial pollination from higher temperature exposed pollen (e). A view of Okra fruits that formed bell-shaped structures (deformed fruits) at elevated temperature treatments; hence seed set and quality of yield hindered (f).

the most susceptible process displaying negative impacts on plant fertility, leading to declined seed set and deformity in fruit formation at (>35°C) exhibiting reduced male fertility. In contrast, stigma receptivity indicated consistency tolerance to merely all temperatures. This study also reports a better understanding of the okra plant's capability to cope with heat stress during reproductive development. To identify potential genetic traits, thus implementing strategies to improve plant heat stress tolerance. This study also delineates the benefits to forestry and agricultural practices shortly as increasing temperatures pose threats to production yield.

Acknowledgment

The support of the University of Guyana, the National Agriculture Research and Extension Institute, and the Guyana School of Agriculture is acknowledged. The Education for Climate Change Adaptation and Mitigation Scholarship provided financial support and World Wildlife Fund Guianas provided a grant for this research. I thank Seeraj Samsundar for statistical guidance and contributions to this research. I thank Mr. Harry of GSA for his support with husbandry practices.

Declaration of interest statement

Authors have no conflict of interest

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Indian J. Plant Genet. Resour. 35(2): 233–240 (2022)
DOI 10.5958/0976-1926.2022.00026.2

RESEARCH ARTICLE

Developmental Pattern and Reproductive Biology of *Nymphaea micrantha* Guill. & Perr. and *Nymphaea nouchali* Burm. f. in Kerala

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(Received: 03 January, 2022; Revised: 13 July, 2022; Accepted: 13 July, 2022)

Water lily (Genus *Nymphaea* L.) has got immense ornamental, medicinal and cultural significance. The study was carried out to understand developmental pattern and reproductive biology of two species in Genus *Nymphaea*, which is fundamental for any crop improvement programme. Flowers were found to be solitary, pedicellate, and complete with various floral whorls in a spiral fashion on the floral axis. Significant variability was observed for various floral characters among the two species evaluated. The flowers were found opened in the morning and closed in the evening hours and the process was repeated on the subsequent days. Blossom life lasted for three days in *Nymphaea micrantha* and four days in *Nymphaea nouchali*. The peak flower production was observed during September to November period. The dehiscence of pollen occurred by the longitudinal splitting of the anthers in both the species and proceeded from the outer whorl to the inner whorl. The pollen grains retained viability only for 30 to 40 minutes. The stigma became receptive 17 hours before flower opening and receptivity was retained up to 32 hours after flower opening. Very low fertility of pollen was observed in both the species used for the study. No fruit or seed development was observed in both the species under study.

Key Words: Anther dehiscence, *Nymphaea*, Reproductive biology, Stigma receptivity, Water lily

Introduction

The genus *Nymphaea* L. or waterlilies consists of about 40-50 species and is widespread in tropical and temperate regions covering vast extents of natural water bodies. (Shashika *et al*., 2016). The genus includes fascinating groups of aquatic plants and forms an important constituent of aquatic flora possessing immense ornamental value for its beautiful and spectacular flowers. *Nymphaea nouchali* is the most widely spread species in Asia (La-ongsri *et al.,* 2009). *Nymphaea nouchali* is commonly known as Indian blue water lily or Indian water lily in English. Though it is not lotus, this water lily is often referred to as 'blue lotus of India' (Slocum, 2005). A lot of synonymies occur for *N. nouchali* (Danin, 2000) and controversy exists among botanists regarding this. In Greek *nymphala* refers to water nymph and *stellata* in Latin means star-shaped. The local name 'Neelathamara' is applicable only to the water lily with bluish flowers, which is *N. stellata* (Nair, 2004).

Nymphaea nouchali exhibits a range of flower colours as a combination of either blue, pale blue, blue violet, violet, pink, purple-red or white (Dassanayake, 1996). Flower colour is considered as an important feature in recognizing intraspecific taxa (Hooker, 1875; Conard, 1905; Slocum *et al.,* 1996) which in turn helps to document the biodiversity richness of a country.

The species in genus *Nymphaea* L. is an important and well-known medicinal plant, widely used in Ayurveda and Siddha systems of medicines. The antidiabetic activity, tumour inhibition property, anti-hepatotoxic effects, analgesic, anti-inflammatory and antimicrobial activities of *N. nouchali* was well studied (Raja *et al.,* 2010). Its tuber can become an economical dietary adjunct functional food full of macro and micronutrients that can help to fight against oxidative stress originating due to modern lifestyle induced metabolic disorders (Anand *et al.*, 2019). The mechanism of anticancer activities of methanolic extract of *N. nouchali* tuber is unravelled by Uddin *et al*. (2020). It is also cultivated for food in Sri Lanka and rhizomes are full of starch and quite tasty when boiled. The roots and rhizomes are considered to be nutritious when eaten either raw or roasted. Flower

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and flower stalks are used as vegetables, green manure and fodder (Slocum, 2005). The flower of *Nymphaea nouchali* is the national flower of Bangladesh and Sri Lanka. The plant is historically and functionally significant since it is associated with our culture and tradition. Despite its immense potential, water lily has received only very little attention from crop improvement workers. Information on developmental patterns and reproductive biology which is fundamental for any crop improvement programme is lacking in this plant. Hence this study was carried out to document the developmental pattern and reproductive biology of two species of Genus *Nymphaea,* from Kerala, India.

Materials and Methods

Field visit conducted to collect the samples of genus *Nymphaea* has resulted in the identification of two species with purple coloured and white coloured flowers, which were collected from Malabar Botanical Garden & Institute for Plant Sciences, Kozhikode, Kerala served as material for the study. The species with purple coloured and white coloured flower were identified as *Nymphaea micrantha* Guill. & Perr. and *Nymphaea nouchali* Burm. f. var. *versicolor* respectively, based on the taxonomic key developed by Ansari and Jeeja (2009). The investigation of developmental and reproductive biology of these two species of *Nymphaea* was carried out in the Department of Plant Breeding and Genetics at the College of Agriculture, Vellanikkara, Kerala Agricultural University, Thrissur, Kerala during 2010 to 2012.

The developmental pattern of flowers, as well as flowering biology, was critically evaluated in the selected species under *ex-situ*. The morphological features of leaves from both species were described by observing fully mature leaves. Various leaf characters were also measured from five fully developed leaves from each species. For studying the growth pattern of flower buds, five flower buds from the two species were tagged immediately after their appearance on the surface of the mud. The growth of flower bud from the visual appearance stage was studied at periodic intervals till opening. The time taken for opening from the visual appearance of the bud was also recorded. The succession of flower formation and the longevity of the flower in each species were also examined. The number of flowers produced each month in both species was recorded and expressed as a percentage of the total number of flowers produced per year. The seasonality of the flowering was then computed. For the convenience of analyzing the seasonal effect on flowering, the whole year was divided into four seasons. The succession of flower formation in the peak period was also recorded. The description of morphological features of both the species was done after examining fresh flowers, on the first day of flower opening.

The colour and appearance of anther were observed with hand lens at hourly intervals from 6 am onwards from a day prior to opening in five fully matured flower buds until the dehiscence of pollen grains, in each species, to find out the time of anther dehiscence (Prasad and Krishnaprasad, 1994). The stigmatic surface was observed for any change in colour or appearance, in the same bud, at hourly intervals to find out the onset of stigma receptivity. Duration of stigma receptivity was also estimated as per standard procedures (Radford *et al.,* 1974). The time and duration of stigma exudates secretion were examined. The presence of stigma exudates on the stigmatic cup or its moist condition was considered as an indication of stigma receptivity. Various insects visiting the flowers were also observed using hand lens.

Pollen grains acetolysis was done as per the method suggested by Nair (1970). The pollen grains subjected to acetolysis were microscopically examined to describe the shape, aperture presence, exine sculpturing and any other special features of the pollen. Pollen size was measured using phase-contrast microscope. The fertility of pollen was assessed on the basis of staining with the acetocarmin-glycerin mixture (Radford *et al*., 1974). The pollen grains which were well stained were classified as fertile and others as sterile. Observations were taken in two microscopic fields. The values were expressed as percentage. The fruit development was checked after the submergence of flower in both the species.

Results and Discussion

Nymphaea L. populations collected during the field survey exhibited morphological variations within the two species studied. The blue-flowered water lily and white or pink flowered are considered two intraspecific taxa of *N. nouchali* and represented as *N. nouchali v*ar. *nouchali* and *N. nouchali* var. *versicolor* respectively (Shashika e*t al.,* 2016). But Ansari and Jeeja (2009) have segregated the white-coloured one as *N. malabarica,* said to be endemic to India. Leaf surface also supported this

grouping. In *Nymphaea micrantha,* the adaxial surface of the leaf was pale green with dark purplish irregular patches and the abaxial surface was green with dark purple spots and a pale purple margin. However, the leaves of *Nymphaea nouchali* were green above and pale green beneath with purple colouration mainly towards the margin. The petiole was glabrous and brownish-green in colour in both species.

The leaves of both species of genus *Nymphaea* under study were simple, orbicular with sub peltate lamina, and deeply cleft near to the petiole base. The leaves were found to be glabrous on both the surfaces with wavy margin and 14 to 15 primary veins prominently raised beneath. The tip of leaf was obtuse or blunt. The petiole was long, slender and submerged in water with lamina floating on the water surface. The length of the petiole varied depending on the depth of water. Dassanayake (1996) also reported similar leaf morphology for *N. nouchali*. *N. nouchali* has given synonyms of *Nymphaea stellata* and *Nymphaea versicolor* by Ansari and Jeeja (2009). Thus, the current study was done considering *N. stellata* as a synonym of *N. nouchali* as indicated by Verdcourt (1989).

The observations recorded on various leaf characters like the length of petiole, lamina and notch, width of lamina at the base, middle and tip at the full expansion stage in the two species of *Nymphaea* are presented in Table 1. A significant difference was observed between the two species for mean length of leaf as well as mean width of leaf at the middle and tip. The *Nymphaea micrantha* was significantly superior to the *Nymphaea nouchali* in all the above-mentioned characters with mean values of 21.97±0.79 cm, 20.76±0.84 cm and 14.24±0.60 cm, respectively. There was no significant difference between the two species in mean length of the notch, width of the lamina at the base and petiole length.

The growth pattern of the flower buds in the two species of *Nymphaea* represented by the mean number of days taken to reach water surface, number of days for flower opening from their visual appearance on the surface of mud, length of pedicel at the time of flower opening and after shedding, length and circumference of the flower bud at maturity, diameter of fully opened flower and blossom life are presented in Table 2. In both species, it took almost six days for the flower bud to reach the water surface. The flower opening occurred nearly three days after the bud reaching the water surface. There was no significant difference between the species in mean pedicel length. Even after the flower opening, the pedicel elongation continued in both the species to an extent of 4 cm. Maximum growth rate of the pedicel was observed on the day just prior to the flower opening (Fig. 1). The increment in pedicel elongation declined after flower opening. The two species differed significantly in size of fully mature flower bud as well as opened flower. The *Nymphaea nouchali* produced longer flower buds and thus larger flowers when compared to *Nymphaea micrantha*. This can be attributed to the superiority of *N. nouchali* in length of flower bud, to the *N. micrantha.* The flowers of both the species were found to be faintly fragrant.

Table 1. Leaf characters of the two species of genus *Nymphaea*

	Length (cm)			Width (cm)		
	Petiole	Lamina	Notch	At the base	Middle	At the tip
N. micrantha	61.25±4.97	21.97±0.79	8.84±0.38	13.81±0.24	20.76±0.84	14.24±0.60
N. nouchali	56.45±2.44	19.71±0.50	8.07±0.27	12.90±0.79	17.68±0.55	11.87±0.49
t-value	NS	2.43*	NS	NS	3.04**	3.05**

* Significant at 5% level ** Significant at 1% level NS - Non significant

Table 2. Growth pattern of flower buds in two species of genus *Nymphaea*

	Days taken to reach water surface	Days to flower opening	Pedicel length (cm)		Size of mature bud		Diameter of flower (cm)	Blossom life (Days)
			At flower opening	On the day of shedding	Length (cm)	Circumference (cm)		
N. micrantha	5.9±0.38	8.8±0.42	24.55±1.28	28.02±1.35	4.43±0.11	6.43±0.36	8.41±0.34	3
N. nouchali	5.4±0.37	8.1±0.41	26.54±1.81	30.47±1.91	5.46±0.25	5.25±0.36	10.19±0.57	4
t-value	NS	NS	NS	NS	3.84**	7.31**	2.66**	#

** Significant at 1% level; NS - Non significant; # Statistical analysis not done since all the values are equal

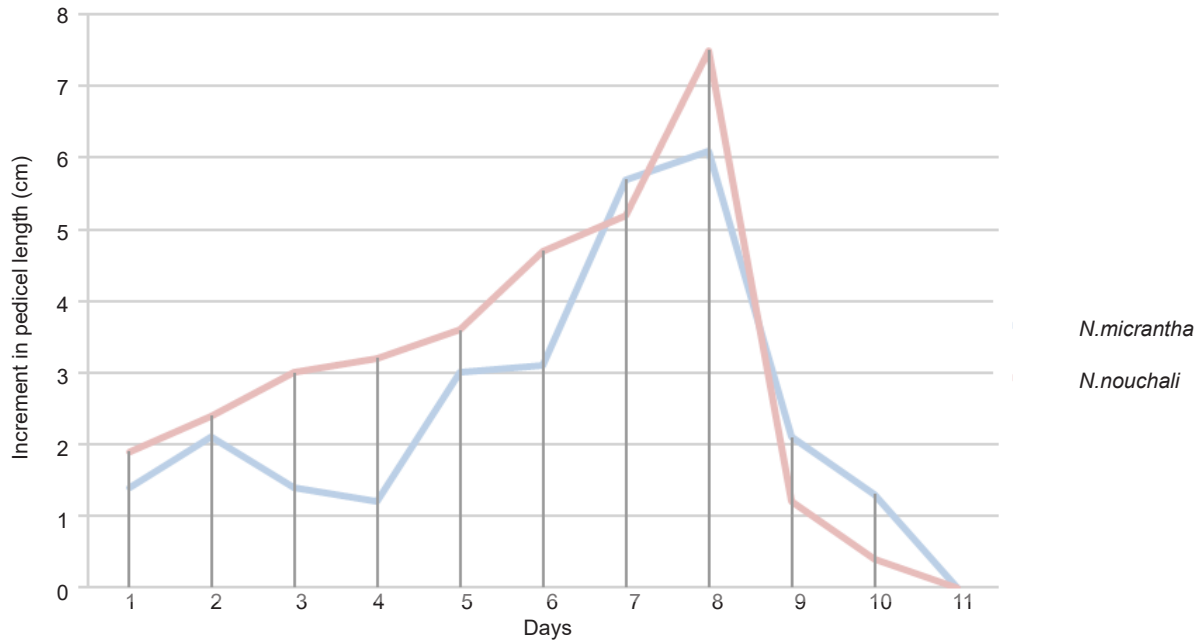

Fig. 1. Increment in pedicel length in two species of genus *Nymphaea*

The blossom life was observed to be three days in *N. micrantha* where as it was four days in *N. nouchali*. The anthesis in both species lasted for three consecutive days. The study by Begum *et al*. (2010) in Bangladesh reported *Nymphaea pubescens* as night bloomer and each flower opens for three consecutive nights whereas *Nymphaea rubra* opened for four consecutive nights and *Nymphaea nouchali* for three consecutive days.

The flowers of both the species opened in the morning and closed in the evening hours and again opened on the next day. The flowers of *Nymphaea micrantha* remained open for three consecutive days, and on the fourth day, it opened partially and bent downwards. On the fifth day, it sank completely into water along with the peduncle. The *Nymphaea nouchali* flowers opened and closed for four consecutive days and sank into water on fifth day. The flower did not dehisce but decayed fully into dark mucilaginous mass after 6-8 days.

The process of blooming began with the opening of the sepals. Depending upon the weather conditions the opening time of flower varied from 7.30 am and extended up to 9.45 am. It took 15-20 minutes for full blooming. The closing time varied from 5.15 pm to 6.15 pm and closing was completed within 10-15 minutes for full closing. Early sunrise favoured early opening of the flower in both species.

The flowers were produced throughout the year. The flower production was more in spring season (September to November). While Begum *et al.* (2010) reported that flowering in *Nymphaea nouchali* and *Nymphaea pubescens* mainly takes place from May to November, whereas flowering in *Nymphaea rubra* occurs round the year. The flowers were produced on an average of 3-4 days in both species. Hence, both these species can be well recommended for water gardens. The floral characters of two species of *Nymphaea* is presented in Tables 3. Flowers were found to be solitary, pedicellate, and complete with various floral whorls in spiral fashion on the floral axis. There were four sepals, 18-21 petals, numerous stamens and 13 to 20 carpels for each flower. The picture of the flower bud and flower on the first day of flower opening in both the species are shown in Fig. 2 and Fig. 3. The length of the petals and stamens showed a gradation in size in both the species under study with the outermost whorls having the largest and the inner most whorls having the smallest units. Each stamen consisted of a filament, anther and a sterile appendage at the tip. The initiation of dehiscence occurred by the longitudinal splitting of the anthers in both the species. The dehiscence of anthers proceeded from the outer whorl to the inner whorl of stamens. The anther dehiscence starts from 10.10 am to 11.30 am on the first day of flower opening and the dehiscence was completed

Table 3. Floral characters of two species of genus *Nymphaea*

Characters	*N. micrantha*	*N. nouchali*	t value
Length of sepal (cm)	4.04±0.09	5.98±0.29	8.13**
Breadth of sepal (cm)	1.77±0.04	1.82±0.11	NS
Angle at the tip	66.66±2.39	61.38±2.86	NS
Length of outer whorl (cm)	3.78±0.07	5.62±0.32	7.56**
Breadth of outer whorl (cm)	1.22±0.03	1.28±0.05	NS
Angle at tip of outer whorl	84.47±2.13	60.96±0.84	7.54**
length of middle whorl (cm)	3.48±0.09	5.28±0.30	7.37**
Breadth of middle whorl (cm)	1.18±0.04	1.18±0.04	NS
Angle at tip of middle whorl	80.43±2.46	55.96±0.67	6.85**
Length of inner whorl (cm)	3.18±0.09	4.73±0.24	7.43**
Breadth of inner whorl (cm)	0.96±0.03	0.93±0.04	NS
Angle at tip of inner whorl	64.10±1.33	46.06±1.74	7.99**
Diameter of stigmatic cup (cm)	2.02±0.05	1.82±0.04	3.24*
No. of carpels/ receptacle	15.20±0.39	19.20±0.86	4.94**
Length of fertile pollen (µm)	39.88±2.62	34.51±1.66	NS
Breadth of fertile pollen (µm)	38.46±2.67	32.14±1.41	NS

** Significant at 1% level NS – Non significant

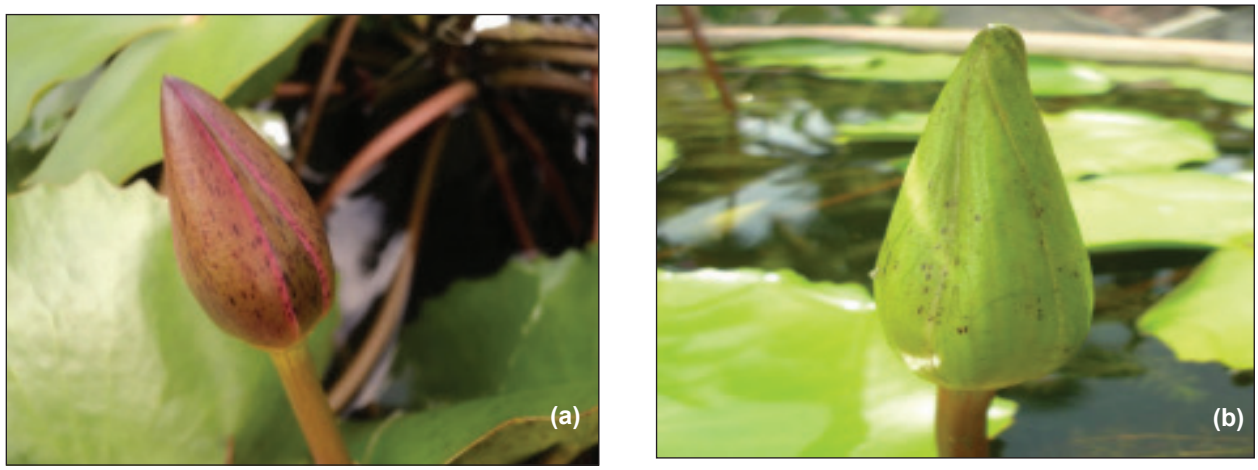

Fig. 2. Flower bud of *Nymphaea micrantha* (a) and *Nymphaea nouchali* (b)

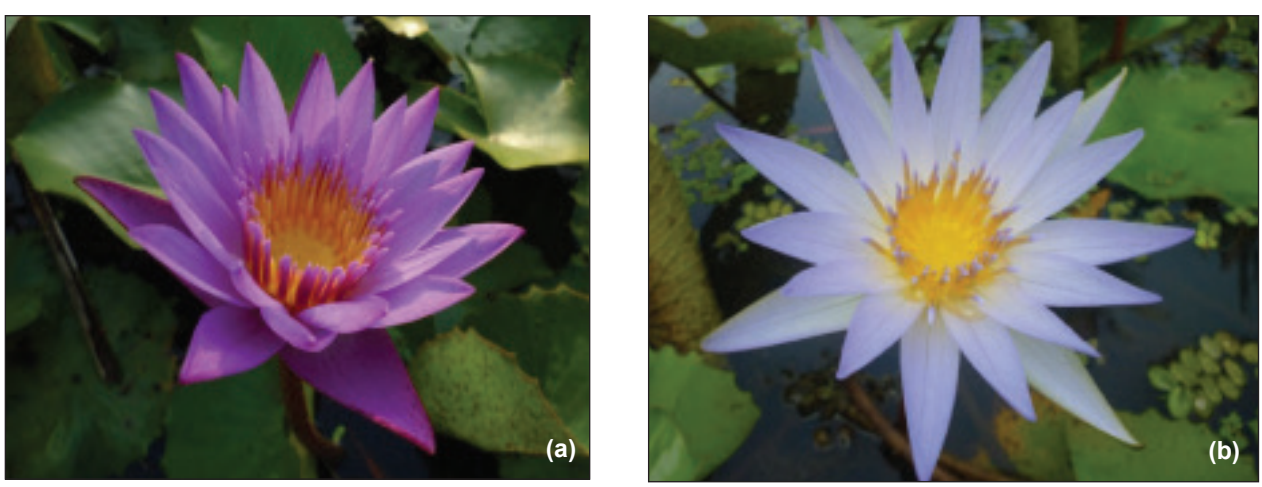

Fig. 3. Flower of (a) *Nymphaea micrantha* and (b) *Nymphaea nouchali*

in 30 to 40 minutes. In both the species, gynoecium was found to be syncarpous, with yellow cup shaped stigma. Stigmatic appendages curved inwards and equal in number to the number of carpels present along the rim of the stigmatic cup. The appendages were more in *N. nouchali* while the stigmatic cup was bigger in *N. micrantha*. The flowers were found to be protogynous. The stigma became receptive 17 hours before flower opening and the receptivity was retained up to 20 hours even after flower opening. The flower morphology of *Nymphaea nouchali* in present investigation is more or less similar to the observations of Dassanayake (1996), Ansari and Jeeja (2009) and Begum *et al.* (2010), but it differed slightly from Conald (1905).

Honey bees, house flies and weevils were found to be the major insects visiting the flowers in both the species. Most of the visiting insects were belonging to Hymenoptera, Odonata and Coleoptera families in *Nymphaea hybrid* (Zhang *et al*., 2021). Several dead insects observed in the stigmatic cup of both the species (Fig. 4). The fertility of pollen grains studied in two species are presented in Table 4. The pollen grains were found to be round, yellow in colour and monocolpate with reticulate exine in both the species. There was no significant difference between the two species in the size of the pollen. Monocolpate pollen grains are reported in *N. nouchali by* Ansari *et al.* (2005) and in *N. micrantha* by Bhowmik and Datta (2012). Length and breadth of fertile pollen was measured as 39.88±2.62 µm and 38.46±2.67 µm in *N. micrantha* 34.51±1.66 µm and 32.14±1.41 µm in *N. nouchali*. A lesser length of 21.12 ± 1.76 µm and the breadth 33.38 ± 2.23 µm was reported by Bhowmik and Datta (2012) in *N. micrantha*. Very low fertility was observed in both the species. Pollen fertility was observed maximum in middle whorl on the second day of flower opening. Pollen viability and stigma receptivity first increased and then decreased with blooming time in *N. hybrid* (Zhang *et al*., 2021). Pollen fertility was observed maximum in middle whorl of stamen, on the second day of flower opening. No fruit or seed development was observed in both species. But excellent fruit set and ellipsoid seeds were reported by Begum *et al.* (2010) in *Nymphaea nouchali* from Bangladesh. Ansari *et al.* (2009) reported the *N. micrantha* species do not set fruits in India. Pollen viability and stigma receptivity are important parameters for successful pollination (*Soares et al*., 2018; Figueiredo *et al*., 2020). The absence of fruit or seed set in these species can be attributed mainly to the very low fertility of pollen grains which needs further detailed investigations. Ansari *et al.* (2003) reported 88% pollen sterility in *Nymphaea micrantha.* High percentage

Table 4. Pollen fertility in two species of genus *Nymphaea*

Colour variant	First day of flower opening (Outer whorl) in %	Second day of flower opening (Middle whorl) in %	Third day of flower opening (Inner whorl) in %
N. micrantha	3.45	5.53	3.33
N. nouchali	5.86	8.27	6.99

(a) **(b)**

Fig. 4. Insects trapped in stigmatic fluid in (a) *Nymphaea micrantha* and (b) *Nymphaea nouchali*

(a) (b)

Fig. 5. New plantlets arising from leaf in (a) *Nymphaea micrantha* and (b) *Nymphaea nouchali*

of pollen sterility inhibits fruit setting in exotic plants. Their study revealed the formation of sterile pollen at tetrad stage as a result of irregular meiotic division. However, the occurrence of hybrid *Nymphaea caerulea* × *Nymphaea micrantha* (or vice versa) produced viable seeds. *The* vegetative propagation from leaf was found to be prominent in both species under study. The new plantlets were found to be arising from the upper portion of mature leaf where petiole touches the lamina (Fig. 5). The new plantlets remain attached to the petiole until petiole decayed. After that, they start growing independently by elongation of roots. Since both the species can be vegetatively propagated, incapability of seed setting does not affect the distribution of plants.

Conclusion

The result of present study is in agreement with the identification of two species in *Nymphaea* populations of Kerala based on leaf and floral characteristics. The developmental pattern and reproductive biology of flowers in *Nymphaea micrantha* and *Nymphaea nouchali* is described. Considering the esthetic, economic and medicinal values, detailed cytological and palynological studies are to be undertaken in these species of *Nymphaea* to unravel the cause of pollen sterility and absence of seed set.

Acknowledgments

The authors are thankful to Department of Plant Breeding & Genetics, College of Agriculture, Vellanikkara, Kerala Agricultural University for providing the facilities for the study. The help rendered by Dr. R. Ansari, Managing Director (Retd.), Malabar Botanical Garden & Institute for Plant Sciences, Kozhikode in the identification of the species studied is gratefully acknowledged.

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Indian J. Plant Genet. Resour. 35(2): 241–249 (2022)
DOI 10.5958/0976-1926.2022.00027.4

RESEARCH ARTICLE

Evaluation of Common Bean (*Phaseolus vulgaris* L.) Germplasm for Agro-Morphological and Yield Traits and Resistance to Bean Common Mosaic Virus (BCMV) in Western Himalayan Kashmir

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(Received: 18 January, 2022; Revised: 04 April, 2022; Accepted: 04 April, 2022)

Kashmir Himalayas abounds in bean genetic resources with adaptive capacity and better quality. In the present study 110 common bean germplasm accessions were evaluated between 2019-2021 for yield traits as well as for BCMV resistance. BCMV is the most common and most destructive disease of beans and can cause a yield loss as high as 100%. Substantial variability was recorded in the material as depicted by broad range and high GCV, PCV and heritability and significant mean squares in ANOVA. Five accessions recorded stable resistance to BCMV while as 11 accessions were moderately resistant. Several genotypes were identified as novel sources of traits in respect of plant architecture, pod and seed traits as well as overall yielding ability. Promising genotypes identified for BCMV resistance as well as other traits of economic importance can be used as stable donors for improving common bean yield and BCMV resistance for sustainable bean farming in the region.

Key Words: BCMV, Common bean, Genetic resources, Resistance, Western Himalayas

Introduction

Common bean (*Phaseolus vulgaris* L.) also called as French bean or Rajmash is an important summer season legume and is an indispensable component of subsistence farming, thus involving low input marginal farmers. There is increasing evidence coming up about the livelihood and health benefits of this crop making it very popular among the farmers due to its quality, nutritional balance and higher biological efficiency. It is an important source of carbohydrate (61.4 %), proteins (17.5-28.5%) and minerals (3.2-5.0%), as well as vitamin C and pro-vitamin A. Besides it contains substantial amount of dietary fiber and minerals like Iron, Potassium, Phosphorus, Magnesium, Copper etc. In India, it is mainly cultivated by the small and marginal hill farmers of Western Himalayan states of Himachal Pradesh, Jammu & Kashmir and Uttarakhand over an area of about 26.75 thousand hectares (Anonymous, 2020). The UT of Jammu and Kashmir (33°17′-37° 20′ N latitude, 73°25′-80°30′ E longitude) harbours great variation in the in common bean genetic resources primarily in the form of traditional landraces and farmers varieties. There is a need to undertake in-depth characterization of the available genetic diversity of common bean to identify trait specific sources that can be used to develop varieties for yield, quality and resilience.

The productivity of common bean is constrained by various intrinsic (pertaining to biology and metabolism) and extrinsic (pertaining to management, climate change, diseases, pest and abiotic stresses) factors. Legumes are invariably low yielding on account of protein energy compensation and energy shifts towards nitrogen fixation. It is also implicated by various biotic and abiotic factors such as diseases, pests, drought, heat and cold stress. Bean Common Mosaic Virus (BCMV) severely affects common bean yield to the extent of complete crop failures and as such demand immediate breeding attention for development of resistant varieties. Collins *et al.* (2019) reported that BCMV is the most common and most destructive and can cause a yield loss as high as 100%. BCMV has been a global bean constraint and is reported from all bean growing areas and has persisted on account of its seed transmission and as such occurs in mild or severe form depending upon the cultivar and environmental conditions.

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BCMV known as Bean Virus-1 or Phaseolus Virus-1 is found in almost all common bean growing areas of the world largely due to its seed-borne nature (Drijfhout 1978; Mckem *et al*., 1992). In India, the occurrence of BCMV was reported for the first time by Yaraguntaiah and Nariani (1963). BCMV infects five major families including *Leguminoceae, Amaranthaceae, Chenopodiaceae, Solanaceae* and *Tetragoniaceae* (Bos and Gibbs, 1995). BCMV causes a variety of symptom pattern and severity depending upon strain, host variety, temperature, management conditions and population of transmitting vectors. There is an urgent need to identify common bean varieties that combine productivity with resilience. The varieties released thus far have not exhibited enough resilience against BCMV and as such there is an urgent need to screen the available common bean germplasm and identify lines that can be promoted as varieties or used as parents in breeding programmes aimed at achieving sustainable BCMV resistance. The conventional field and greenhouse based screening protocols can be combined with molecular tools to identify QTLs/ genes governing BCMV resistance.

In Western Himalayan states of J&K, Himachal Pradesh and Uttarakhand BCMV occurs in mild or severe forms. The major races identified have been NL1, NL1n, NL7 and NL7n (Kapil *et al.,* 2011; Hamid *et al.,* 2016). The varieties released thus far both at state level as well as under AICRP MULLaRP have not exhibited enough resilience against BCMV. Rigorous filed and greenhouse screening coupled with molecular tools can help identify QTLs/ genes governing BCMV resistance. The present study was aimed at characterizing a core set of common bean representing diverse market classes for phenological and yield traits, besides screening the set against BCMV resistance response across multiple environments under field and greenhouse conditions.

Materials and Methods

Plant material: The Plant material used for the present study comprised of a set of 110 diverse genotypes of common bean including 5 checks (Shalimar Rajmash-1, Shalimar Rajmash-2, Shalimar French Bean-1, Arka Anoop, Arka Sharath (Fig. 1). The planting material was from diverse sources representing a variety of growth habits, use categories and seed and pod variants, comprising both local landraces and exotic genotypes. It represented major market classes such as small seeded red, large kidney red, yellow, white navy, white great northern beans, black beans and chocolate beans. Among the checks Shalimar Rajmash-1, Shalimar Rajmash-2 and Shalimar French Bean-1 are varieties released by SKUAST-K, Arka Anoop and Arka Sharath are varieties released by IIHR-Bangalore.

Experimental site: The experimental plant material was planted at four environments between 2019-2021, including four different locations of Kashmir (Fig. 2), besides under greenhouse conditions. The environments were:

Environment-1 (E4 2020 and E5 2021): Research field of Division of Genetics and Plant Breeding FoA Wadura (34^{o} 17′ N and 74^{o} 33′ E at an altitude of 1594 masl). The soil of the experimental site at Wadura is a typical inceptisol with clay loam texture.

Environment-2 (E1 2019): Dryland Agricultural Research Station, Rangreth (33^{o} 98’ N and 74^{o} 79’ E at an altitude of 1640 masl). The soil is an inceptisol with silty clay texture

Environment-3 (E2 2020): KVK Ganderbal, Shuhama. The site is located at an altitude of 1588 masl (34^{o} 12’ N and 74^{o} 46’E). The soil is an alfisol with clay loam soil.

Environment-4 (E3 2020): Farmer’s field at Saloora. The site is located at an altitude of 1619 masl (34^{o} 12’ N and 74^{o} 46’E) with clay loam soil.

Environment-5 (E6 2020): Greenhouse FoA Wadura. The soil mix was derived from the research field of the Faculty with addition of sand and vermicompost to ensure better growth.

Experimental design: The experiment was set up in an augmented block design (Federer, 1956).The design comprised of five (5) blocks, each containing twenty one (21) test genotypes and five (5) checks. Thus in each block there were 26 entries. The checks in each block were randomly allocated for estimation of error as well as standard errors of comparison.

Data recording on agronomic traits: In order to study the magnitude of variability for yield and its contributing traits among 110 lines, data was recorded for 10 agro-morphological traits at Research field of Division of Genetics and Plant Breeding FoA Wadura. The traits were days to flowering, days to maturity, plant height, number of pods per plant, pod length, seeds per pod, seed length, seed breadth, 100-seed weight and seed yield per plant.

Field and greenhouse screening of genotypes for BCMV

resistance: For screening BCMV resistance response, common bean genotypes were screened against bean common mosaic virus at 3 weekly stages beginning from 25 days after emergence stage. Screening was done in the open field trials at three locations *viz*., FoA Wadura, KVK Ganderbal, and Farmer's field at Saloora and scored as per scale developed by Horsfall and Barrat (1945) and Drijfout (1978) and the genotypes were grouped into various response classes by scale developed by CIAT (Mills and Silbernagel, 1992).

Under greenhouse screening, the leaves at trifoliate stage were inoculated by sap method as proposed by Kelly *et al.* (1995) by extracting the sap from plants showing BCMV symptoms by macerating symptomatic leaves with a mortar and pestle in cold phosphate buffer, pH 7.0 for stabilizing the inoculum. The primary leaves were inoculated using conventional leaf rub method following abrasion. The inoculum was maintained on ice until the inoculation process was completed. The plants were monitored for 15 days and evaluated for symptomatic variation using the 1-9 scale given by Mills and Silbernagel (1992). 1-2 Resistant; 3-4 moderately resistant; 5-6 moderately susceptible; 7 Susceptible; 8-9 Highly susceptible.

The leaves were critically observed for the established symptoms of BCMV such as mosaic, stunting, curling, discoloration and chlorosis. The observed results were further validated by leaf observations against sunlight as well as under microscope to identify chlorotic patches. The per cent disease incidence (PDI) of BCMV was estimated for each accessions based on data obtained from each seasons at two locations and the final mean of PDI was calculated based on average PDI mean of five seasons and across the location. The per cent disease incidences of BCMV for each accession were computed by using the formula given below.

Statistical analysis: The mean data from first experiment was analyzed for estimation of basic statistics and analysis of variance (ANOVA) for assessing variation according to the expected value of mean square as described by Federer (1956) and Federer and Searle (1976). The analysis of augmented design was carried out by SPAD (Statiscal package for augmented design) platform developed by IASRI (Rathore *et al*., 2004). The genetic variability components estimated were phenotypic coefficient of variance (PCV), genotypic coefficient of variance (GCV), broad sense heritability (h^2), expected genetic advance (GA) and expected genetic advance as percent of mean (GAM) (Burton and Devane, 1953). Phenotypic and genotypic coefficients of variation (PCV and GCV) for each trait were computed as PCV = $\{\sqrt{\sigma^2}P/ \text{ mean}\}$ x 100, GCV = $\{\sqrt{\sigma^2}G/ \text{ mean}\}$ x 100 as per (Burton 1952). Heritability was estimated as $h^2 = \{\sigma^2G/\sigma^2P\}$ x 100 as per method of Lush (1940) and further classified into low, medium and high (Robinson 1966). Genetic advance as percent of mean was estimated as GA= k x h^2 x σP as per Johnson *et al.* (1955). The standard value of k at 5 % selection intensity is taken as 2.06. Homogeneity of variance was tested before statistical analysis on the adjusted pooled means as per procedure of Levene (1960). Principal components (PC) were computed to determine the patterns of variation and the genetic relationship existing between accessions in the collection using XLSTAT Version 2021.4 (Addinsoft Inc.).

Results and Discussion

Variability for agro-morphological traits

Plant height: Plant height ranged widely as the genotypes characterized comprised both determinate bush and indeterminate pole types (Table 1). The mean plant height was 68.13cm with the minimum and maximum values of 36.70 cm and 213.00 cm respectively. Genotypes such as WB-923, Arka Komal WB-352, KDFB-38, WB-1441 were having smaller plant height (< 40 cm), whereas genotypes WB-1282, WB-222, GL-1, WB-451 were taller in height (>150 cm).

Days to flowering and maturity: Among phenological traits, the mean value recorded for days to flowering (Table 1) was 43.62 days with lowest value recorded for genotypes WB-662, WB-1319, WB-1249, WB-651 (37 days) followed by WB-662, WB-1644, N-2, N-4, WB-185, WB-923 (38 days) whereas the genotypes WB-1518, WB-1455, WB-901, WB-206, Arka Komal, WB-252, WB-1282, WB-451, WB-1189, WB-222 were late in flowering (53 days). Days to maturity also had a broad range with a few genotypes such as WB-222, GL-1 and GL-2 as late maturing. The mean value recorded was 81.03 days with lowest value recorded for WB-1319, WB-651, WB-923 (68 days) followed by WB-662, WB-1634, N-1, N-4, WB-185, WB-923, SFB-1 (73 days) whereas the genotypes GL-1, GL-2, WB-222 matured in 99 days.

Similar results in common bean have been reported under Himalayan conditions in earlier studies of Rana *et al.* (2015), Iram Saba *et al.* (2017), Rani Shama (2019) and Sofi *et al.* (2020). The lower variation range in

Table 1. Variability parameters for 10 quantitative traits in common bean

Trait	Mean	Min	Max	PCV	GCV	Heritability	Genetic advance (% of mean)
DF	43.62	36.00	58.00	10.82	10.05	0.86	19.17
DM	81.03	68.00	99.00	13.88	13.06	0.88	25.16
PH (cm)	68.13	36.70	213.00	25.12	23.18	0.89	46.06
NPP	14.40	4.80	47.66	24.46	24.76	0.92	46.36
PL (cm)	11.26	9.10	17.33	9.08	7.91	0.83	15.52
SPP	4.63	3.00	7.34	17.67	15.75	0.77	28.03
SL (mm)	12.60	7.83	17.42	8.56	7.18	0.75	13.23
SB (mm)	7.38	4.57	9.74	7.27	5.09	0.67	10.03
100SW (g)	33.55	15.75	55.72	14.76	14.39	0.98	29.80
SYPP (g)	22.27	19.56	89.54	21.15	19.77	0.79	34.42

DF=days to flowering, DM=days to maturity, PH= plant height, NPP= number of pods/plant, PL= pod length, SPP= seeds per pod, SL= seed length, SB= seed breadth, 100SW= 100-seed weight, SYPP=seed yield per plant

Table 2. Analysis of augmented design for morphological, maturity and yield traits in 110 genotypes of common bean

Trait	df	DF	DM	PH	NPP	PL	SPP	SL	SB	100SW	SYPP
Block	4	9.63**	12.11	53.12**	52.08**	3.81*	0.36*	0.02	0.01	5.46 *	27.74
Among Genotypes	109	22.30**	126.54**	16016.91**	86.11**	9.08 **	0.67**	0.04**	0.03**	177.63**	111.18**
Among Test entries	104	60.27**	466.72**	260.11**	79.29**	8.76**	8.50**	0.04**	0.05**	209.09**	142.85**
Among Checks	4	24.15**	10.95**	16422.51**	373.38**	5.26**	0.61**	0.06**	0.01**	236.60**	57.58**
Test entries v/s checks	1	87.12**	10.23	54451.13**	278.65**	98.23**	0.82**	0.06**	0.01**	119.51**	75.44**
Error	16	3.09	14.56	17.26	6.80	1.49	0.15	0.02	0.02	2.05	23.30

DF=days to flowering, DM=days to maturity, PH= plant height, NPP= number of pods/plant, PL= pod length, SPP= seeds per pod, SL= seed length, SB= seed breadth, 100SW= 100-seed weight, SYPP=seed yield per plant

Fig. 1. Diversity in common bean germplasm under evaluation

maturity traits is due to the fact that farmer's preference for short duration varieties has driven selection for early maturity. However the indeterminate types are more preferable under maize based intercropping systems and are invariably late in maturity. The estimates of PCV, GCV, heritability and genetic advance (% of mean) were higher for plant height followed by days to maturity and days to flowering. The heritability estimates were 0.89, 0.88 and 0.86 respectively for these traits.

Pod traits: The mean value recorded for number of pods per plant was 14.40 with highest value recorded in WB-864 (47.66) followed by WB-371 (40.30), WB-1634 (33.10) and WB-341 (29.20) whereas the lowest value was recorded for KDFB-38 (4.80). Similarly, the mean value recorded for pod length was 11.26 cm with highest pod length recorded for WB-195 (17.33) followed by N-7 (15.10 cm), WB-970 (13.90 cm), WB-923 and WB-966 (13.40 cm each) whereas the lowest value was recorded for WB-651 (9.10 cm) and KDFB-38 (9.10 cm). The mean value recorded for seeds per pod was 4.63with highest value recorded for WB-371 (7.34) followed by N-1 (6.12) and WB-1634 (5.26) whereas the lowest value was recorded for WB-6 and N-5 (3.00). The estimates of PCV, GCV and genetic advance (% of mean) were higher for number of pods per plant followed by seeds per pod and pod length. The heritability estimates were 0.92, 0.77 and 0.83 respectively for these traits (Table 1). Similar results have been reported for pod traits under Kashmir conditions by Sofi *et al.* (2014), Iram Saba *et al.* (2017), Rani Shama (2019), Asmat Ara (2019) and Sofi *et al.* (2020). Since common bean pods are a favorite vegetable in Kashmir and as such, pod traits also assume importance in varietal development (Sofi *et al.,* 2020).

Seed traits: Mean value recorded for seed length was 12.60 mm with highest value recorded for WB-1441 (17.42 mm) followed by WB-832 (17.32 mm) and WB-966 (17.10 mm) whereas the lowest value was recorded for WB-435 (9.08 mm). The seed breadth was also substantially variable, with mean value of 7.38 mm. The highest value for seed breadth was recorded for WB-1249 (9.47 mm) followed by WB-966 (8.97 mm) and WB-970 (8.91 mm) whereas the lowest value was recorded for WB-603 (4.72 mm). For 100-seed weight, the mean value recorded was 33.55 g with highest value recorded for WB-6 (55.72 g) followed by WB-967 (55.00 g) and WB-966 (54.60 g) whereas the lowest value was recorded for GL-3 (14.76 g) . Seed yield per plant was also highly variable with broad range. The mean value recorded was 22.27 g with highest value recorded for N-1 (89.54 g) followed by N-4 (79.14.00g) and WB-1634 (68.27 g) whereas the lowest value was recorded for KDFB-38 (19.56 g). The estimates of PCV, GCV and genetic advance (% of mean) were higher for seed yield per plant and low for seed length and seed breadth (Table 1). The heritability estimates for these traits were 0.98 (100-seed weight) followed by seed yield per plant (0.79), seed length (0.75) and seed breadth (0.67). Similar results have been reported for seed traits under Kashmir conditions by Sofi *et al.* (2014), Iram Saba *et al.* (2017), Choudhary *et al.,* 2018, Asmat Ara (2019). Sofi *et al.* (2020) also reported high heritability estimates for seed traits ranging from 76.69 % for seeds per pod to as high as 98.36 % for 100-seed weight. The smaller difference between the GCV and the PCV, with GCV invariably smaller than PCV indicates that the observed variation and expression of traits is mainly due to genetic factors while larger difference in case of traits like seed length and seed breadth indicate the greater role of the environment. Higher heritability for most of the traits indicated that these traits may be governed by additive genes and use of simple selection methods may bring significant improvement.

In the present study 105 test entries along with 5 checks were evaluated in an augmented block design for agro-morphological and yield traits. The analysis of variance revealed that the net block effect (after eliminating treatment effect) was non-significant for days to maturity, seed length, seed breadth and seed yield per plant. The mean square due to genotype effect (after eliminating block effect), test entries as well as checks was significant for all the 10 traits studied. The test genotype effect was significant for days to flowering, days to maturity, days to mid pod fill, and days to pod fill and non-significant for days to pod set (Table 2). The mean square due to test vs. check comparison was significant for all the traits except days to maturity. The substantial variability in the material as indicated by significant mean sum of squares for genotypes, test entries as well as checks can be effectively utilized for development of varieties in various use category classes (such as dry, snap and shelled) based on maturity, pod and seed characters. Substantial variability in common bean germplasm comprising both local landraces and material from national and international gene banks has been reported in Western Himalayan conditions by

Fig. 2. Representative range of BCMV symptoms recorded in the field

Rana *et al.* (2015), Iram Saba *et al.* (2017), Choudhary *et al.* (2018), Rani Shama (2019) and Sofi *et al.* (2020). Pod and seed traits have also been reported to be highly heritable traits (Nienhuis and Singh, 1988, Sofi *et al.,* 2014, Langat *et al.,* 2019) and as such there is ample scope for improvement using appropriate selection strategies in target environments.

Identification of novel trait sources for plant architecture, maturity and yield components: Despite the fact that major focus of present study was on assessment of variability for phenological and yield traits and screening bean germplasm for BCMV resistance, nevertheless, an attempt was made to identify novel trait sources for phenological and yield traits. Out of the 110 germplasm lines that were evaluated across locations, many novel trait specific genotypes were identified (Supplementary Fig. 2) based on early maturity (WB-923, WB-662, WB-956, SFB-1, N-4, WB-1319, N-2 and WB-1455), pod length (N-7, WB-970, WB-195, WB-956), number of pods per plant (WB-864, WB-371, WB-1634), seeds per pod (WB-371, WB-901, WB-451, N-7, WB-258, WB-1282), 100-seed weight (WB-6, WB-257, WB-216, WB-967, WB-1439, N-7, WB-1492), pod shattering resistance (WB-216,WB-1129, WB-1006, WB-46 and WB-206), stay green (WB-216), erect plant type (N-1). There is an urgent need to identify common bean genotypes with novel trait combinations that improve their fitness in farming systems as well as combine productivity with resilience and quality. This requires in-depth characterization of natural variation in available genetic diversity for productivity and water stress adaptive traits. Already a large number of accessions are collected and conserved in gene banks that need to be characterized in depth. In national gene bank (NBPGR), a total of 172 accessions of bean germplasm from Jammu and Kashmir are conserved. (Source: pgrportal.nbpgr.ernet.in).

The trait-specific germplasm is highly imperative for genetic enhancement of crop varieties for various traits. In the present study various unique germplasm for specific traits Identified hold a great promise in improving common bean crop in the region. More importantly, the BCMV resistant genotypes that were identified in the present study could be used as stable sources of resistance as the genotypes have exhibited resistance response under diverse screening environments. The early maturing genotypes which mature within 70-75 DAS could play an important role in sustaining dry bean production as the early maturity will be an important motivation for farmers to bring more area under bean cultivation. Some of the highly productive pole type lines could be utilized for maize-bean intercropping that is prevalent in Western Himalayan region. Overall a few genotypes had combined advantage of higher yield and BCMV resistance including N-1, WB-1634, and WB-1129.

Symptom spectrum of BCMV under field conditions: Bean common mosaic virus causes a variety of symptom pattern and severity depending upon strain, host variety, temperature and management conditions as well as population of transmitting vectors. In the present study, a variety of diverse symptoms ranging from complete death of the plant to varied degree of mosaic, leaf discoloration, leaf chlorosis, yellowing, leaf crinkling, puckering were observed (Fig. 2).

Vectors recorded under field conditions: BCMV is laterally transmitted through viruleferous aphids (Hampton, 1975; Morales and Castaño, 1987). Various viruliferous aphids such as bean aphid (*Aphis fabeae*), Pea aphid (*Acyrthosiphon pisum*) and green peach aphid (*Myzus persiceae*) can transmit BCMV and increase infection upto 100% (Galvez and Morales, 1989). However, the aphids transmit the virus in a non-persistent manner. In the present study two aphid species were

recorded under field conditions at FOA Wadura that might have a possible role in horizontal transmission of BCMV that needs to be investigated further. However, green peach aphid (*Myzus persiceae*) was not recorded under field conditions.

Mean reaction pattern of common bean genotypes to BCMV across four screening environments: Pooled across environments (filed as well as green house) the phenotypic screening of 110 common bean genotypes against BCMV resistance (Supplementary Table 1) revealed that out of 110 genotypes screened, only five genotypes (less than 5%) genotypes namely WB-1129, WB-216, WB-206, N-10 and WB-45 were resistant, 11 genotypes (10%) namely N1, WB-1691, WB-916, WB-765, WB-1131, WB-1680, WB-1256, Arka Anup, WB-1710, WB-1634, WB-373 were moderately resistant, 62 genotypes (56%) were moderately susceptible, 29 genotypes (26%) were susceptible and three genotypes (less than 3%) genotypes namely N-2, SR-1 and WB-1698 were highly susceptible with BCMV score of >8 (Supplementary Table 1 and Table 4).

Earlier various studies have been undertaken in western Himalayan conditions for screening common bean reaction to BCMV. In Himachal Pradesh, Sharma *et al.* (2006) evaluated ninety four common bean accessions comprising of landraces, exotic collections and recommended cultivars against three strain groups (I, IV, VI) of BCMV and reported two exotic varieties TopCrop and Amanda highly resistant to all the three strain groups. Sharma *et al.* (2008) screened a larger core set of 397 common bean accessions of diverse origin revealing the presence of effective sources of resistance against bean common mosaic potyvirus strains prevalent in Himachal Pradesh; 21accessions *viz*., KR 7, KR 225, KR 295, KRC 4, KRC7, KRC 11, KRC 12, KRC 13, KRC 16, KRC 22, Amanda, Black Turtle Soup, Contender, Hans, Great Northern UI 123, Improved Tender Green 40031, Jubila, Kentucky wonder, Monroe, Premier and Sanilac were found resistant to NL-1n and NL-7n strains. In Western Himalayan Kashmir, local and exotic common bean genotypes have also been screened earlier BCMV resistance based on field and greenhouse evaluations. Wani *et al.* (2017) and Rani Shama (2019) revealed various resistant lines (WB-399, WB-640, WB-359, WB-375, WB-494, WB-933, WB-939 and also WB-335) were found phenotypically resistant. The result was later ascertained by molecular analysis by using different microsatellite markers. Rani Shama (2019) had also identified genotypes WB-206, WB-1129, WB-642 and Arka Komal) as resistant. The present study has thus validated the resistance response of various genotypes such as WB-1129, WB-206, WB-642 and Arka Komal as well as the susceptible response of genotypes SR-1, KDFB-37, WB-1187, WB-1644, WB-662, WB-1446, WB-435, and WB-482 reported earlier.

Conclusion

In the UT of J&K, a large diversity of beans largely in form of landraces is grown. These landraces have evolved under natural and farmer-driven selection process and are locally very well adapted. In terms of varietal output, three varieties namely Shalimar Rajmash-1 and Shalimar Rajmash-2 (dry beans) and Shalimar French Bean-1 (Snap) have been released by

Table 4. Status of reaction of 110 common bean genotypes against BCMV in filed screening

Reaction type	Genotypes	Number of genotypes
Resistant	WB-206, WB-1129, WB-216, N-10, WB-45	5
Moderately Resistant	N-1, WB-1691, WB-916, WB-765, WB-1131, WB-1680, WB-1256, Arka Anup, WB-1710, WB-1634, WB-373	11
Moderately susceptible	WB-6, WB-22, WB-92, WB-112, WB-185, WB-191, WB-195, WB-218, WB-371, WB-401, WB-418, WB-429, WB-451, WB-630, WB-634, WB-642, WB-643, WB-650, WB-651, WB-665, WB-716, WB-832, WB-846, WB-901, WB-955, WB-957, WB-967, WB-1006, WB-1136, WB-1137, WB-1184, WB-1185, WB-1255, WB-1274, WB-1282, WB-1310, WB-1318, WB-1436, WB-1441, WB-1446, WB-1492, WB-1496, WB-1518, WB-1554, WB-1560, WB-1574, WB-1587, WB-1643, WB-1677, WB-1678, WB-1682, N-11, N-5, N-7, N8, GLY-1, KDR-98, DARS-10, DARS-38, SR-2, SFB-1, Arka Sharat	62
Susceptible	WB-46, WB-83, WB-115, WB-333, WB-352, WB-487, WB-489, WB-565, WB-662, WB-869, WB-923, WB-956, WB-1171, WB-1182, WB-1249, WB-1319, WB-1435, WB-1437, WB-1455, WB-1485, WB-1644, N-4, GLP-1, KDFB-81, KDFD-3, KDR-63, KDR-97, DARS-10-1, DARS-43	29
Highly susceptible	N-2, SR-1 and WB-1698	3

SKUAST-Kashmir and two varieties are in pipeline. In the present study, an attempt was made to characterize a set of 110 genotypes revealing substantial variability in agro-morphological and yield traits indicating usefulness of the material for further selection. A number of genotypes with novel traits related to plant architecture, pod and seed traits have been identified. Moreover, a number of stable sources of resistance to BCMV were identified that can be used in a planned hybridization programme to combine productivity with resilience. Out of the present material several desirable lines have been already submitted to ICAR and IC numbers received that will ensure conservation and utilization of these valuable genetic resources in future rajmash breeding programmes in the country. Moreover, a large number of crosses have been developed based on this study that are at different stages and are being currently evaluated for identification of desirable segregants.

Acknowledgements

The authors acknowledge the support of various collaborators for germplasm evaluation.

*Supplementary Table or Figure mentioned in the article are available in the online version.

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Supplementary Table 1. Screening score of 110 common bean genotypes for resistance to BCMV across six screening environments

Genotype	2019 (E1)	2020 (E2)	2020 (E3)	2020 (E4)	2021 (E5)	2020 (E6)	Mean Score
WB-6	5.3	6.1	5.4	4.7	4.2	5.5	5.20
WB-22	6.3	4.5	7.0	6.5	4.7	7.0	6.00
WB-45	2.5	1.3	2.0	1.8	3.7	5.0	2.72
WB-46	7.9	8.0	7.3	7.2	3.5	9.0	7.15
WB-83	7.1	7.9	6.3	6.0	8.0	8.0	7.22
WB-92	7.3	7.0	7.8	7.3	4.8	7.0	6.87
WB-112	5.6	5.0	5.0	5.3	5.4	7.0	5.55
WB-115	7.3	7.6	7.5	7.0	8.3	7.0	7.45
WB-185	5.6	6.4	5.7	5.5	4.7	5.0	5.48
WB-191	5.0	4.7	5.0	5.3	8.3	5.0	5.55
WB-195	6.3	7.0	6.3	6.0	5.5	6.0	6.18
WB-206	1.8	0.5	2.0	1.7	2.0	2.0	1.67
WB-216	1.6	0.5	1.5	1.3	2.8	2.0	1.62
WB-218	5.2	5.0	4.7	5.0	6.2	6.0	5.35
WB-333	6.7	7.0	6.7	6.0	9.0	7.0	7.07
WB-352	6.8	6.5	7.5	7.3	8.8	6.0	7.15
WB-371	5.9	6.6	6.8	7.0	4.7	3.0	5.67
WB-373	4.8	4.0	5.0	5.3	5.8	5.0	4.98
WB-401	6.0	7.0	6.5	5.7	6.5	5.0	6.12
WB-418	6.5	6.6	6.0	6.5	5.8	7.0	6.40
WB-429	6.3	6.0	7.5	6.7	5.8	5.0	6.22
WB-451	6.3	6.6	6.7	6.0	5.3	6.0	6.15
WB-487	7.5	7.4	8	7.7	5.7	7.0	7.22
WB-489	7.5	7.6	7.5	8.0	4.5	7.0	7.02
WB-565	6.8	7.6	6.5	6.0	8.8	7.0	7.12
WB-630	6.2	6.6	6.3	5.8	6.1	6.0	6.17
WB-634	5.9	6.6	5.7	5.5	4.7	6.0	5.73
WB-642	6.7	6.0	6.7	7.0	6.2	7.0	6.60
WB-643	6.3	6.4	6.3	6.5	7.3	6.0	6.47
WB-650	6.8	7.0	6.7	6.3	6.5	7.0	6.72
WB-651	6.4	7.8	6.0	5.7	8.1	6.0	6.67
WB-662	7.6	7.4	7.7	7.2	6.2	8.0	7.35
WB-665	6.7	7.6	6.0	6.0	4.7	7.0	6.33
WB-716	7.0	7.5	6.7	6.7	6.8	7.0	6.95
WB-765	4.3	4.0	5.0	5.0	4.2	5.0	4.58
WB-832	6.4	6.1	6.3	6.0	1.0	7.0	5.47
WB-846	5.8	5.7	6.7	5.7	8.0	6.0	6.32
WB-869	8.0	8.0	8.0	7.0	7.8	9.0	7.97
WB-901	6.0	6.3	5.7	6.0	6.0	6.0	6.00
WB-916	4.2	5.3	4.7	5.0	4.2	4.0	4.57
WB-923	8.0	7.8	7.5	7.7	5.9	9.0	7.65
WB-955	6.7	7.0	7.0	6.7	5.7	6.0	6.52
WB-956	6.8	7.4	6.0	6.0	8.7	8.0	7.15
WB-957	7.1	7.5	6.5	6.5	5.0	8.0	6.77
WB-967	6.5	8.0	5.5	4.7	5.0	8.0	6.28
WB-1006	5.3	6.5	6.0	3.5	6.3	5.0	5.43
WB-1129	1.3	1.4	1.7	1.0	1.0	1.0	1.23

Genotype	2019 (E1)	2020 (E2)	2020 (E3)	2020 (E4)	2021 (E5)	2020 (E6)	Mean Score
WB-1131	4.6	6.6	4.5	3.2	5.8	4.0	4.78
WB-1136	4.7	6.0	4.8	4.0	7.5	4.0	5.17
WB-1137	5.5	6.0	5.3	4.8	7.0	6.0	5.77
WB-1171	7.3	7.7	6.0	7.0	6.7	8.0	7.12
WB-1182	7.2	7.8	7.0	7.0	8.7	7.0	7.45
WB-1184	6.4	7.4	5.8	5.5	5.5	7.0	6.27
WB-1185	6.4	7.6	6.5	5.5	7.0	6.0	6.50
WB-1249	7.1	7.8	6.5	6.3	8.0	8.0	7.28
WB-1255	6.9	6.5	7.0	7.0	7.2	7.0	6.93
WB-1256	5.3	4.0	5.0	5.0	5.2	5.0	4.92
WB-1274	6.6	7.0	6.3	6.0	5.8	7.0	6.45
WB-1282	6.5	6.1	7.0	7.0	5.8	6.0	6.40
WB-1310	6.6	7.0	6.3	6.0	8.2	7.0	6.85
WB-1318	6.3	7.0	6.0	6.0	6.1	6.0	6.23
WB-1319	7.4	7.9	6.8	7.0	7.0	8.0	7.35
WB-1435	7.4	7.0	7.0	8.0	7.3	8.0	7.45
WB-1436	6.0	6.0	6.0	6.0	7.0	6.0	6.17
WB-1437	7.0	7.0	6.5	6.3	7.3	8.0	7.02
WB-1441	6.9	8.0	7.0	5.7	6.2	7.0	6.80
WB-1446	6.5	7.1	6.7	5.3	7.8	7.0	6.73
WB-1455	7.1	7.8	6.3	6.5	7.3	8.0	7.17
WB-1485	7.1	7.5	6.5	6.3	7.3	8.0	7.12
WB-1492	6.2	7.3	6.5	6.0	6.8	5.0	6.30
WB-1496	5.1	5.0	5.3	6.3	7.0	4.0	5.45
WB-1518	6.1	6.5	5.7	5.3	4.7	7.0	5.88
WB-1554	6.6	7.0	6.3	6.0	6.4	7.0	6.55
WB-1560	6.6	7.6	6.0	5.7	7.4	7.0	6.72
WB-1574	6.2	7.4	5.7	5.7	7.3	6.0	6.38
WB-1587	5.6	5.5	6.7	4.7	7.3	6.0	5.97
WB-1634	5.1	6.3	4.8	4.3	4.3	5.0	4.97
WB-1643	5.7	6.1	5.7	4.0	4.0	7.0	5.42
WB-1644	6.9	8.0	6.7	5.0	7.8	8.0	7.07
WB-1677	6.0	5.8	5.7	5.5	4.5	7.0	5.75
WB-1678	6.5	6.6	6.0	6.4	8.5	7.0	6.83
WB-1680	4.6	4.3	4.3	5.0	5.8	5.0	4.83
WB-1682	6.3	6.5	6.0	5.7	5.3	7.0	6.13
WB-1691	4.7	4	5.0	4.7	2.7	5.0	4.35
WB-1698	8.0	8.0	7.3	7.7	9.0	9.0	8.17
WB-1710	4.9	4.7	5.0	5.0	5.0	5.0	4.93
N1	3.2	3.1	4.2	2.6	2.8	3.6	3.25
N10	2.1	2.7	1.8	3.0	1.0	3.0	2.27
N11	6.3	4.5	6.3	5.8	5.5	6.5	5.82
N2	7.6	8.1	7.8	7.7	8.8	8.2	8.03
N-4	7.0	7.9	6.7	6.5	8.8	7.0	7.32
N5	6.2	5.3	5.8	6.2	6.0	6.7	6.03
N7	4.7	5.6	5.2	6.1	6.0	6.1	5.62
N8	5.4	6.1	6.4	5.4	6.6	5.8	5.95

Genotype	2019 (E1)	2020 (E2)	2020 (E3)	2020 (E4)	2021 (E5)	2020 (E6)	Mean Score
GLY-1	7.1	7.4	7.0	7.0	5.7	7.0	6.87
GLP-1	8.5	6.9	7.6	8.1	8.4	8.4	7.98
KDFB-81	7.6	7.0	7.8	7.5	7.9	8.0	7.63
KDFD-3	7.6	7.9	7.7	7.7	7.4	7.0	7.55
KDR-63	7.2	7.0	8.0	7.7	9.0	6.0	7.48
KDR-97	6.9	7.0	6.7	7.0	7.5	7.0	7.02
KDR-98	6.9	7.5	6.0	6.0	6.0	8.0	6.73
DARS-10	6.7	7.8	5.8	6.3	6.8	7.0	6.73
DARS-10-1	7.4	7.5	7.7	7.5	6.9	7.0	7.33
DARS-38	6.8	6.8	6.0	6.3	7.1	8.0	6.83
DARS-43	7.2	7.6	6.7	6.3	7.3	8.0	7.18
SR-1	7.9	8.0	7.5	7.2	9.0	9.0	8.10
SR-2	6.3	7.0	6.0	5.2	7.3	7.0	6.47
SFB-1	5.4	6.3	5.0	4.3	6.1	6.0	5.52
Arka Anup	5.4	6.3	5.0	4.5	2.3	6.0	4.92
Arka Sharat	4.4	5.7	5.6	5.7	5.7	6.4	5.58

$$PDI\ (\%) = \frac{\text{Total number of infected plants per plots}}{\text{Total number of plants per plots}} \times 100$$

Supplementary Fig. 1. View of field experiments across locations and years

Supplementary Fig. 2. Promising genotypes with better trait expression

Indian J. Plant Genet. Resour. 35(2): 250–256 (2022)
DOI 10.5958/0976-1926.2022.00028.6

RESEARCH ARTICLE

Analysis of Genetic Diversity and Survey of QTLs for Grain Yield under Drought Stress in Drought Tolerant Rice Landraces Using DTY QTL-linked Markers

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(Received: 18 March, 2022; Revised: 03 June, 2022; Accepted: 04 June, 2022)

Genetic diversity in a set of 60 rice genotypes were analyzed using 27 random and 13 grain yield under drought (DTY) quantitative trait loci (QTLs)-linked simple sequence repeats (SSR) markers. The average gene diversity polymorphism information content were 0.53 and 0.46, respectively. The presence of DTY QTLs for grain yield under drought stress was predicted using peak marker in comparison to the positive checks. The DTY QTL $qDTY_{12.1}$, amplified by the peak marker RM 28048, was found in 43.3% of genotypes, and $qDTY_{2.2}$was detected in only 6.67 % of genotypes using peak marker RM279. Two germplasm accessions, IC389895 and DT 2, possessed maximum number of DTY QTLs and absent is IC525099. Phylogenetic analysis revealed four distinct clusters within the germplasm. Overall, the evaluated genotypes presented a rich source of diversity and contain valuable DTY QTLs which can be utilized in rice genetic enhancement program for drought tolerance.

Key Words: Drought tolerance, DTY QTL, Genetic diversity, Rice, SSR marker

Introduction

Rice is the staple food for more than half of the global population. Worldwide, around 40% of rice cultivated area comes under rainfed environments (Singh *et al*., 2017). Rainfed rice production systems can be classified into lowland and upland. In Asia rainfed lowland rice area is about 46 million hectares which is almost 30 % of the total world rice area (Maclean *et al*., 2002). Large rice areas in Bangladesh, Cambodia, Myanmar, Nepal, and Thailand, India, Indonesia, Laos, and Vietnam fall under rainfed ecology (CGIAR, 1998). Rice cultivation in rainfed areas face severe challenges of drought and flooding. Drought will possibly become more frequent due to adverse effect of climate change (World Bank, 2009). Therefore, drought-prone rice systems in rainfed areas require stress tolerant rice varieties along with improved crop management strategies. Recent efforts to identify major QTLs with a large and consistent effect on grain yield under drought condition have marked a valid strategy (Bernier *et al.*, 2007; Kumar *et al.*, 2007; Venuprasad *et al.*, 2009; Vikram *et al.*, 2011). Coordinated drought breeding programmes have exhibited a significant positive trend genetic gain for grain yield over the years under both drought stress as well as favorable irrigated control conditions (Kumar *et al*., 2021).

The rice landraces traditionally cultivated by the farmers, contain high level of genetic diversity, and can serve as potential genetic resources for improvement of yield under biotic and abiotic stresses (Choudhury *et al*., 2013). As the major element of germplasm, genetic diversity is a natural source for rice breeding to encounter existing food requirements (Reig *et al*., 2016). Identifying rice cultivars with drought tolerance and high level of genetic diversity will aid in rice improvement programme for rainfed drought-prone ecologies. The present study was conducted with a set of 57 rice landraces, selected earlier from a larger set of germplasm based on tolerance to drought, an objective to analyze genetic diversity and predict the presence of DTY QTLs using SSR markers. The knowledge thus generated will help in utilization of these cultivars for rainfed rice improvement.

Materials and Methods

Plant materials and DNA isolation

A set of 60 rice genotypes (Table 1) that include 57drought tolerant rice cultivars and three check varieties

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viz. Sadabahar, Vandana and Sahbhagi Dhan were used in the study (Table 1). The germplasm set was selected based on tolerance to vegetative stage drought tolerance from 258 rice cultivars mostly from the eastern Indian region (data not shown). During genotyping drought tolerant checks such as N22, IR64Drt1, Vandana, Apo and Way Rarem were used. Total genomic DNA was collected from five-day old seedlings were isolated using a modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The concentration of genomic DNA was checked in Nanodrop system 2000c (Thermo, USA), and the actual concentration was adjusted to 50 ng μL^{-1}.

SSR markers used

A total of 40 SSR markers, 27 random and 13 DTY QTL-linked were used in the study (Supplementary Table 1). The random markers were sampled from GCP panel of 50 SSRs (http://gramene.org/markers/microsat/50_ssr.html). The primer sequences and annealing temperatures are available at Gramene Database (http:// www.gramene.org).

Polymerase chain reaction and scoring of amplicons

Polymerase chain reaction (PCR) was carried out in a total volume of 10 µL reaction having 50 ng of template DNA, 0.5 µM of each forward and reverse primers, 0.2 mM of dNTPs, 1X PCR buffer with 20 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Thermo Fisher Scientific, USA). PCR was done in a thermocycler (Veriti^{TM}, Applied Biosystems) using following conditions: 5 min at 94°C; 35 cycles of 45 s at 94°C 45 s at annealing temperature and 45 s at 72°C; followed by 5 min at 72°C. PCR amplicons were visualized in 3-4% agarose gels stained with SYBR Safe DNA gel stains (Invitrogen) using a UV-transilluminator. During scoring, the band with the lowest molecular weight was assigned allele number 1 and the progressively heavier bands were scored incrementally. DNA molecular weight standards were used in agarose gels to determine amplicon sizes.

Screening of DTY QTLs

Out of 14 DTY QTLs linked SSR markers, nine peak markers were used to screen for the presence of drought tolerant yield QTLs (Supplementary Table 1). The size of amplified fragment of each genotype under study was compared with the amplicon size of the respective DTY QTL positive check to score for the presence of DTY QTLs.

SSR Data analysis

The summary statistics of the SSR markers matrices such as number of alleles, major allele frequency, observed heterozygosity, expected heterozygosity gene diversity and polymorphism information content were estimated. The phylogenetic tree was constructed using Roger's

Table 1. List of rice genotypes used in the study

	IC/ collector No.		IC/ collector No.		**IC/ collector No.**
1	RSR2/JLM-9	21	IC-389895	41	IC568239
2	RSR2/JLM-12	22	IC-419206	42	IC568303
3	RSR2/JLM-34	23	IC-454009 sathi	43	IC568288
4	RSR2/JLM-40	24	IC-454256	44	IC568236
5	RSR/SKY-22	25	IC-454498X BALA	45	IC568294
6	KP-2036	26	IC-454599 KRISHNA	46	IC568223
7	SKSS-05	27	IC-454372X	47	IC568237
8	SKSS-06	28	IC-454628 PTB 28	48	IC568262
9	SKSS-09	29	IC-454634 PTB 30	49	RO-46
10	SKSS-12	30	IC-459347 AC-45	50	JCR-1875
11	SKSS-15	31	IC515116	51	SKY-67
12	PTP/DC-40	32	IC515117	52	SKY-68
13	PTP/DC-43	33	IC525099	53	DT 2
14	PTP/DC-61	34	IC538351	54	DT 10
15	DPS/OPD-179	35	IC538653	55	DT 13
16	DPS/OPD-187	36	IC75844	56	DT 14
17	NR-25 JADAN	37	IC548644	57	DT 35
18	NR-26 CHHATRI DHAN	38	IC568278	58	Vandana
19	NR-31 LAL DHAN	39	IC568250	59	Sahbhagi Dhan
20	IC-264006	40	IC568228	60	Sadabahar

1972 genetic distance and UPGMA method using Power Marker V3.25 (Liu and Muse, 2005). The phylogenetic tree was constructed using Tree View Software.

Results and Discussion

Screening for DTY QTLs

The molecular weight of each of the peak SSR markers associated with each DTY QTLs: $qDTY_{1.1}$, $qDTY_{1.2}$, $qDTY_{2.2}$, $qDTY_{2.3}$, $qDTY_{3.1}$, $qDTY_{3.2}$, $qDTY_{4.1}$, $qDTY_{6.1}$ and $qDTY_{12.1}$) was compared with the amplicon size of the respective DTY QTL-positive check (Supplementary Fig. 1). The positive checks are mentioned in Table 2. The genotypes under study were found to possess more than one DTY QTLs. Two germplasm, IC389895 and DT 2, possessed maximum number of DTY QTLs, while 11 genotypes such as Sadabahar ($qDTY_{3.1}$), NR-31LALDHAN ($qDTY_{3.2}$), IC568288 ($qDTY_{3.2}$), IC538653 ($qDTY_{3.2}$), DT10 ($qDTY_{6.1}$), DT13 ($qDTY_{2.3}$), RSR2/

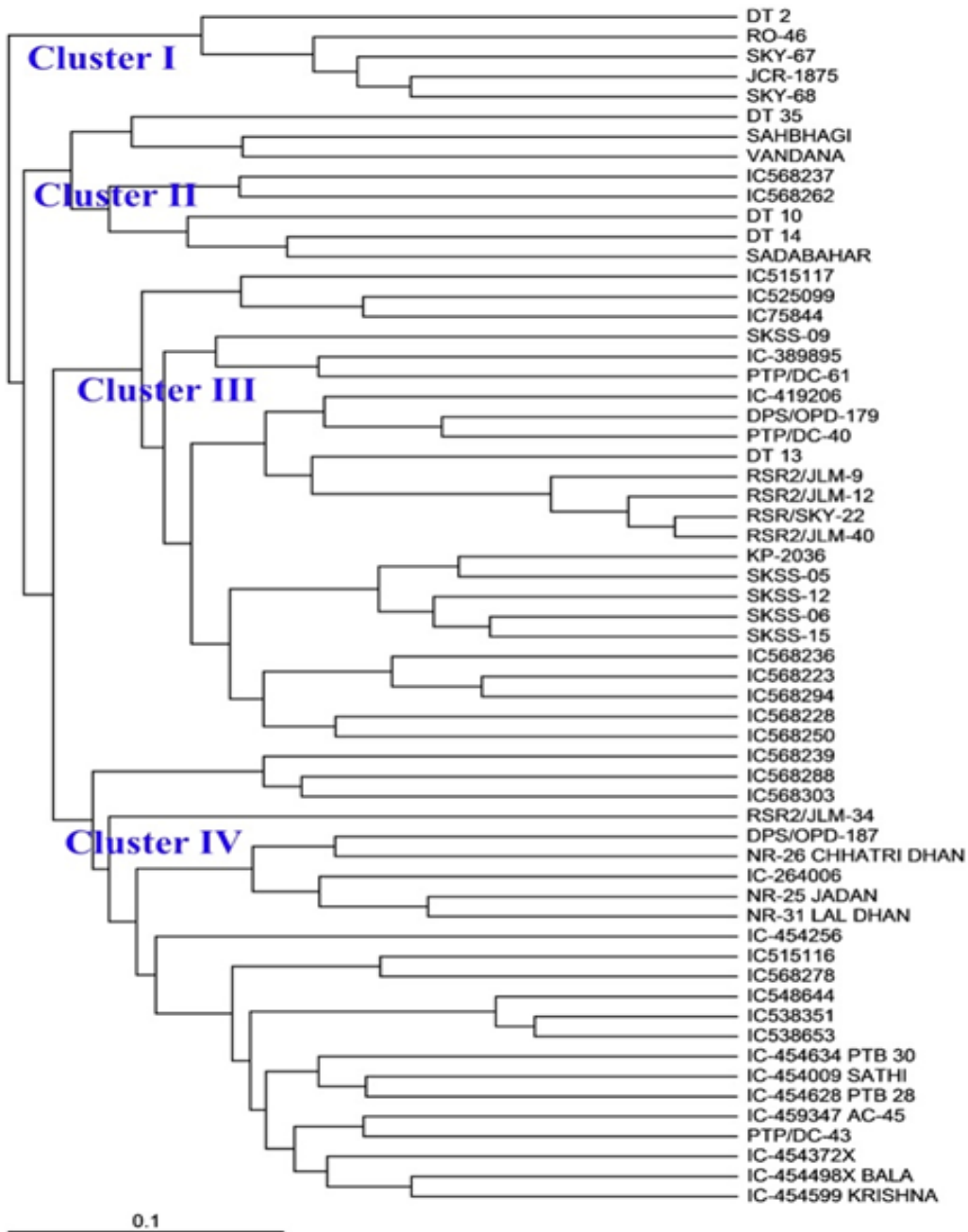

Fig. 1. UPGMA clustering of 60 rice genotypes using 40 SSR markers

JLM-9 ($qDTY_{12.1}$), RSR2/JLM-40 ($qDTY_{12.1}$), RSR/SKY-22 ($qDTY_{12.1}$) and RSR2/JLM-12 ($qDTY_{12.1}$) were found to possess only one DTY QTL (Supplementary Fig. 2). IC-454599 and DPS/OPD-187 showed specific amplicon for $qDTY_{1.1}$ using both the linked markers RM431 and RM 11943. Only four genotypes: SKSS-12, SKSS-15, IC568250 and DT-2 showed $qDTY_{2.2}$-specific amplicon using RM 279. Specific amplicon for $qDTY_{12.1}$ was noted in 43.3% of the total genotypes using RM28048. A total of 21 genotypes were found to possess $qDTY_{6.1}$ based on the banding pattern of linked marker RM589. DTY QTLs such as $qDTY_{2.3}$ $qDTY_{3.1}$, $qDTY_{3.2}$ and $qDTY_{4.1}$ were predicted to be present in 26.66%, 21.67%, 28.33% and 18.33% of the total genotypes, respectively.

The DTY QTLs such as $qDTY_{2.2}$ and $qDTY_{3.1}$ had a consistent effect across seasons under lowland drought stress conditions (Dixit *et al*., 2012; 2014). Under severe drought stress, a grain yield advantage of 0.8-1.0 t ha^{-1} was reported the variety IR64 through the introgression of two QTLs ($qDTY_{2.2}$ and $qDTY_{4.1}$) (Swamy *et al*., 2013). The *qDTY12.1* was also reported to show a consistent effect across environments for high grain yield under drought stress at reproductive-stage in the background of popular high-yielding but drought-susceptible variety (Mishra *et al*., 2013). Therefore, screening of the genotypes for the possible presence of DTY QTLs will further generate scope for validation and utilization of the promising rice germplasm in drought breeding.

Table 2. DTY QTL survey in sixty rice genotypes

DTY QTLs (peak marker)	Positive check	Promising genotypes	Number (%)
$qDTY_{1.1}$ (RM431)	N22	RSR2/JLM-34, DPS/OPD-187, IC-264006, IC-454009, IC-454256, IC-454498X BALA, IC-454599 KRISHNA, IC-454372X, IC568239, IC568303, IC568288, RO-46, DT 35	13 (21.67%)
$qDTY_{1.1}$ (RM11943)	N22	SKSS-12, PTP/DC-43, DPS/OPD-187, NR-25 JADAN, NR-26 CHHATRI DHAN, IC-389895, IC-454599, IC-459347 AC-45, IC515116, IC538351, IC75844, IC568278, IC568236, IC568294, JCR-1875, SKY-68, DT 2	17 (28.33%)
$qDTY_{1.2}$ (RM3825)	N22	SKSS-05, SKSS-06, SKSS-15, PTP/DC-43, DPS/OPD-187, IC-454256, IC-454498X BALA, IC-454599, IC-454628 PTB 28, IC-454634 PTB 30, IC-459347 AC-45, RO-46, SKY-67, DT 2	14 (23.33%)
$qDTY_{2.2}$ (RM279)	IR64 Drt-1	SKSS-12, SKSS-15, IC568250, DT 2	4 (6.67%)
$qDTY_{2.3}$ (RM 3212)	Vandana	RSR2/JLM-9, SKSS-12, PTP/DC-40, DPS/OPD-179, IC-389895, IC-419206, IC515117, IC568239, IC568303, IC568236, IC568294, IC568223, SKY-68, DT 2, DT 13, DT 14, Vandana	16 (26.66%)
$qDTY_{3.1}$ (RM520)	Apo	KP-2036, SKSS-09, SKSS-12, PTP/DC-61, DPS/OPD-179, IC-389895, IC515117, IC568239, IC568294, IC568223, IC568237, DT 14, Sadabahar	13 (21.67%)
$qDTY_{3.2}$ (RM22)	Vandana	KP-2036, SKSS-06, SKSS-09, PTP/DC-40, PTP/DC-61, DPS/OPD-179, NR-25 JADAN, NR-31 LAL DHAN, IC-264006, IC-389895, IC-419206, IC-459347 AC-45, IC538351, IC538653, IC548644, IC568228, IC568239, IC568288, SKY-67, SKY-68, DT 2, DT 35, Vandana	23 (38.33%)
$qDTY_{4.1}$ (RM518)	IR64 Drt-1	PTP/DC-61, IC-389895, IC-454256, IC-454628 PTB 28, IC568278, IC568250, IC568303, IC568237, SKY-67, DT 2, DT 35	11 (18.33%)
qDTY6.1 (RM589)	Vandana	SKSS-05, SKSS-09, PTP/DC-40, DPS/OPD-179, DPS/OPD-187, NR-26CHHATRI DHAN, IC-389895, IC-454498XBALA, IC-45KRISHNA, IC-454372X, IC-454634 PTB 30, IC515116, IC515117, IC75844, IC568288, IC568223, IC568262, SKY-68, DT 10, DT 35, Vandana	21 (35%)
$qDTY_{12.1}$ (RM28048)	Way Rarem	RSR2/JLM-9, RSR2/JLM-12, RSR2/JLM-34, RSR2/JLM-40, RSR/SKY-22, SKSS-05, SKSS-09, PTP/DC-40, PTP/DC-43, PTP/DC-61, IC-389895, IC-419206, IC-454009, IC-454498X BALA, IC568239, IC568288, IC568236, IC568294, IC568223, IC568237, IC568262, RO-46, JCR-1875, SKY-67, SKY-68, DT 2, Shabhagi Dhan	26 (43.33%)

SSR polymorphisms

The summary statistics of the SSR markers is presented in Supplementary Table 2. A total of 133 alleles were detected in 60 rice genotypes using 40 SSR markers. The number of alleles per locus ranged from 2 (RM495, RM507, RM514, RM338, RM455, and RM44) to 5 (RM518, RM6368, and RM118) with an average of 3.325 alleles/locus. The average numbers of alleles per locus observed in this study correspond to other previous studies (Cho *et al*., 2000; Anand *et al*., 2012; Nachimuthu *et al*., 2015). The level of polymorphism among the present genotypes was assessed by calculating PIC values of the 40 SSR loci. The PIC value ranged from 0.138 (RM 161) to 0.677 (RM 16030), with an average 0.457. These values are also consistent with PIC value (0.475) obtained in Anupam *et al*. (2017) while studying genetic diversity of rice germplasm from the state of Tripura, India. Altogether, 17 SSR markers including 8 tightly linked DTY QTL markers (RM 279, RM 22, RM 520, RM 3212, RM 518, RM 11943, RM 3825 and RM16030) recorded PIC values >0.50. A PIC value higher than 0.5 indicates higher polymorphism and are extremely useful in distinguishing the polymorphism rate of markers at specific locus (Botstein *et al*., 1980; Dewoody *et al*., 1995). Present study indicated that the DTY QTLs linked SSR markers were more informative than that of random SSR marker. Yadav *et al.* (2013) also indicated that average genetic diversity at genomic loci assessed by QTL-linked markers is more than that revealed by random markers and reported that study of diversity based on drought QTLs revealed the existence of greater variability at the functional regions of the genome. Gene diversity of expected heterozygosity value was highest (0.726) for DTY QTL linked SSR marker (RM16030) which generated the maximum 5 bands while, the lowest value (0.142) was noted with random SSR marker (RM161). The average gene diversity in the present germplasm was 0.525. The gene diversity value is a fundamental measure of genetic variation in a population and describes the proportion of heterozygous genotypes expected under Hardy-Weinberg equilibrium (Nei, 1973). Gene diversity obtained in the present study is comparable to those reported in previous studies such as 0.52 in Nachimuthu *et al*. (2015) and 0.54 in Choudhary *et al*. (2013). Major allele frequency is maximum with marker RM161 (0.925), minimum with 0.350(RM 16030, RM 3825). The most common allele at each locus ranged from 92% (RM161) to 35% (RM16030 and RM 3825). The average major allele frequency in the present study was higher as compared to the previous studies on Indian rice varieties (Upadhyay *et al*., 2012) and Korean landraces (Li *et al*., 2014).

Genetic Structure Analysis

The assessment of genetic diversity of germplasms is one of the potential approaches which lead to identification of diverse parents for designing effective breeding strategy for hybridization (Sajib *et al*., 2012; Nachimuthu *et al*., 2015). SSR markers have remarkable potential to discriminate between rice genotypes compared to other molecular markers (Xiao *et al.,* 1996). The genetic distance based UPGMA clustering divided the genotypes into four major clusters (Fig. 1). The Cluster I consisted of five genotypes with all the nine DTY QTLs except $qDTY_{3.1}$. Eight genotypes including 3 check varieties were grouped in Cluster II. The Cluster III was comprised of 24 genotypes. In this cluster, RSR/SKY-22 and RSR2/JLM-40 had the lowest genetic distance of 0.05 followed by RSR2/JLM12 - RSR2/JLM40 (0.09). All the nine DTY QTLs were detected within the members of Cluster III. Altogether 23 genotypes grouped in Cluster IV, and eight DTY QTLs, excluding $qDTY_{2.2}$, were found within this cluster. In Cluster IV, the highest genetic distance was recorded between PTP/DC-61 and Vandana (0.80) followed by PTP/DC 40 and DT 2 (0.71). Importance of clustering of these genotypes using both DTY QTL linked markers and random SSR markers can be easily visualized as the released varieties could be improved for drought tolerance using distantly related germplasm without narrowing the genetic base, for instance, rice variety Sadabhar was found to distantly related to IC568250 (genetic distance = 0.65). IC568250 was found to have specific banding for multiple QTLs ($qDTY_{2.2}$ and $qDTY_{4.1}$) and thus can be crossed with Sadabahar to generate superior drought tolerant progenies without narrowing the genetic base.

Conclusion

In the present study, screening of sixty drought tolerant rice genotypes for the possible presence of drought QTLs and the level of genetic diversity provided the scope of utilizing of these genotypes in rice varietal improvement programme for drought stress. The study of genetic diversity among the genotypes enhanced the possibility of using a more diverse donor for drought stress, minimizing the effect of genetic erosion through widening of genetic

base. Use of DTY QTL-linked markers in both screening of the genotypes as well as studying of genetic diversity among these genotypes gave valuable information about the amplification profile of these markers that can be useful for monitoring introgression of DTY QTLs in drought susceptible varieties.

*Supplementary Table or Figure mentioned in the article are available in the online version.

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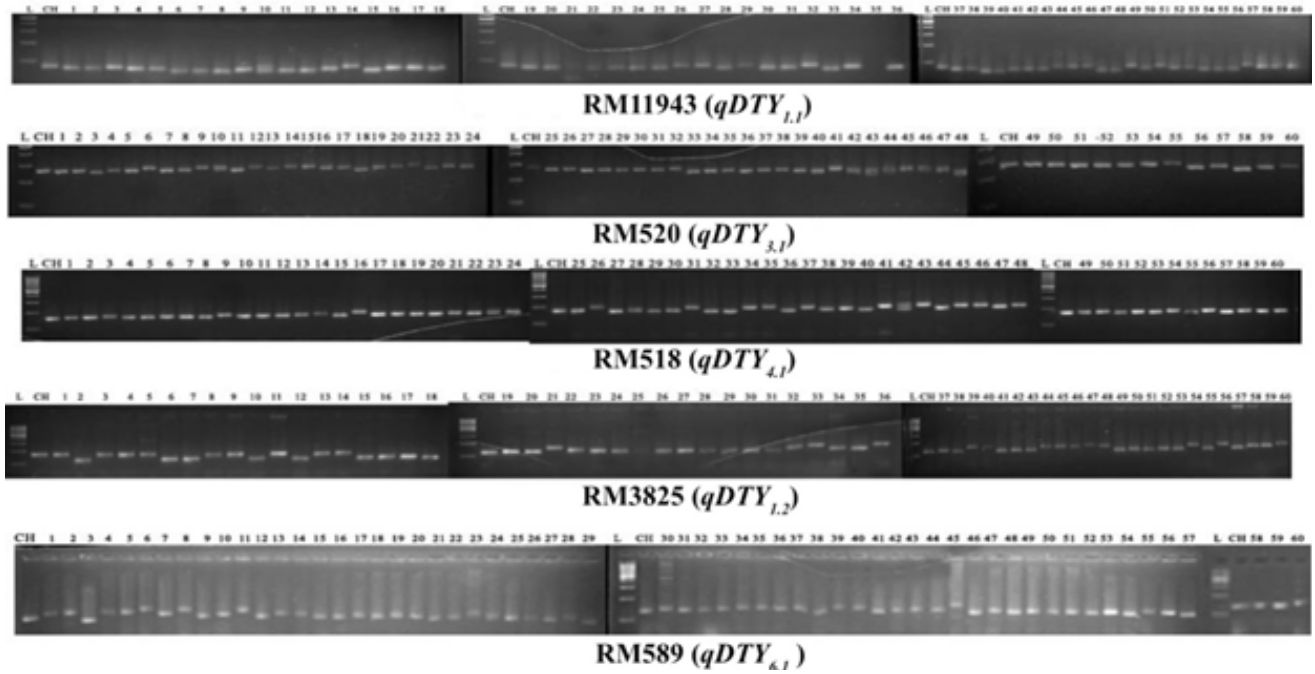
SupplementaryTable 1. SSR markers used in the study

Marker	Chromosome	Random/ DTY QTL-linked	Reference/Source
RM 237	1	Random	See http://gramene.org/markers/microsat/50_ssr.html for details of random SSR markers
RM 283	1	Random	
RM 495	1	Random	
RM 154	2	Random	
RM 408	2	Random	
RM 452	2	Random	
OSR 13	3	Random	
RM 514	3	Random	
RM 338	3	Random	
RM124	4	Random	
RM 161	5	Random	
RM 507	5	Random	
RM413	5	Random	
RM 162	6	Random	
RM133	6	Random	
RM118	7	Random	
RM125	7	Random	
RM 455	7	Random	
RM 152	8	Random	
RM 447	8	Random	
RM284	8	Random	
RM 433	8	Random	
RM 44	8	Random	
RM 316	9	Random	
RM215	9	Random	
RM 484	10	Random	
RM 536	11	Random	
RM431	1	$qDTY_{1.1}$	Vikram *et al*., 2011
RM 11943	1	$qDTY_{1.1}$	Vikram *et al*., 2011
RM 3825	1	$qDTY_{1.2}$	Sandhu *et al*., 2014
RM 279	2	$qDTY_{2.2}$	Swamy *et al*., 2013, Palanog *et al*., 2014
RM1367	2	$qDTY_{2.3}$	Palanog *et al*., 2014, Sandhu *et al*., 2014
RM 3212	3	$qDTY_{2.3}$	Palanog *et al*., 2014, Sandhu *et al*., 2014
RM 520	3	$qDTY_{3.1}$	Dixit *et al*., 2014
RM16030	3	$qDTY_{3.1}$	Dixit *et al*., 2014
RM 22	3	$qDTY_{3.2}$	Vikram *et al.,* 2011
RM 518	4	$qDTY_{4.1}$	Swamy *et al*., 2013
RM16368	4	$qDTY_{4.1}$	Swamy *et al*., 2013
RM 589	6	$qDTY_{6.1}$	Venuprasad *et al*., 2012
RM28048	12	$qDTY_{12.1}$	Bernier *et al*., 2007
RM28130	12	$qDTY_{12.1}$	Bernier *et al*., 2007

Supplementary Table 2. Genetic diversity information of 40 SSR loci in 60 rice genotypes

Marker	Major allele frequency	Allele No	Gene Diversity	Observed heterozygosity	Polymorphism information content
RM 152	0.74	3.00	0.41	0.30	0.37
RM 154	0.53	3.00	0.61	0.02	0.54
RM 162	0.63	3.00	0.50	0.02	0.42
OSR13	0.54	4.00	0.55	0.12	0.45
RM 161	0.93	4.00	0.14	0.02	0.14
RM 237	0.81	3.00	0.33	0.03	0.30
RM 283	0.48	3.00	0.61	0.00	0.53
RM 316	0.48	3.00	0.59	0.03	0.51
RM 408	0.72	3.00	0.42	0.00	0.34
RM 447	0.38	4.00	0.66	0.10	0.59
RM 452	0.52	3.00	0.52	0.03	0.40
RM 495	0.58	2.00	0.49	0.00	0.37
RM 507	0.92	2.00	0.15	0.00	0.14
RM 514	0.85	2.00	0.26	0.00	0.22
RM 536	0.53	4.00	0.62	0.08	0.55
RM 3825	0.35	4.00	0.71	0.00	0.65
RM 11943	0.41	4.00	0.69	0.02	0.64
RM 279	0.57	4.00	0.61	0.00	0.56
RM 520	0.46	4.00	0.65	0.08	0.58
RM 518	0.47	5.00	0.67	0.03	0.62
RM 22	0.45	3.00	0.64	0.00	0.57
RM28130	0.73	3.00	0.42	0.00	0.37
RM28048	0.68	4.00	0.49	0.17	0.44
RM 3212	0.44	4.00	0.67	0.05	0.61
RM1367	0.50	4.00	0.55	0.00	0.44
RM16368	0.50	3.00	0.57	0.00	0.48
RM16030	0.35	5.00	0.73	0.05	0.68
RM431	0.65	3.00	0.51	0.00	0.44
RM118	0.48	5.00	0.68	0.00	0.63
RM124	0.72	4.00	0.45	0.00	0.41
RM125	0.63	3.00	0.50	0.00	0.41
RM133	0.58	3.00	0.56	0.00	0.49
RM284	0.52	3.00	0.59	0.00	0.50
RM 338	0.75	2.00	0.38	0.00	0.31
RM 433	0.45	3.00	0.64	0.00	0.57
RM 484	0.50	3.00	0.53	0.00	0.42
RM215	0.57	3.00	0.52	0.00	0.41
RM413	0.57	4.00	0.59	0.00	0.52
RM 455	0.75	2.00	0.38	0.00	0.31
RM 44	0.57	2.00	0.49	0.00	0.37
Mean	0.58	3.33	0.53	0.03	0.46



Supplementary Fig. 1. Representative agarose gel photographs using SSR markers linked to different DTY QTLs

S.No.	Genotype	qDTY1.1 (RM111943)	qDTY1.1 (RM431)	qDTY1.2 (RM3825)	qDTY2.2 (RM279)	qDTY2.3 (RM3212)	qDTY3.1 (RM520)	qDTY 3.2 (RM22)	qDTY 4.1 (RM518)	qDTY 12.1 (RM28048)	qDTY 6.1 (RM 589)
1	RSR2/JLM-9									√	
2	RSR2/JLM-12									√	
3	RSR2/JLM-34		√			√				√	
4	RSR2/JLM-40									√	
5	RSR/SKY-22									√	
6	KP-2036						√	√			
7	SKSS-05			√						√	√
8	SKSS-06			√				√			
9	SKSS-09					√	√	√		√	√
10	SKSS-12	√			√		√				√
11	SKSS-15			√	√						
12	PTP/DC-40							√		√	
13	PTP/DC-43	√		√		√				√	
14	PTP/DC-61					√	√	√	√	√	
15	DPS/OPD-179						√	√			√
16	DPS/OPD-187	√	√	√							√
17	NR-25 JADAN	√						√			
18	NR-26 CHHATRI	√									
19	NR-31 LAL DHAN							√			
20	IC-264006		√					√			
21	IC-389895	√				√	√	√	√	√	√
22	IC-419206					√		√		√	√
23	IC-454009 SATHI		√			√				√	
24	IC-454256		√	√					√		
25	IC-454498X BALA		√	√		√				√	√
26	IC-454599	√	√	√		√					√
27	IC-454372X		√			√					√
28	IC-454628 PTB 28			√		√			√		
29	IC-454634 PTB 30			√		√					√
30	IC-459347 AC-45	√		√		√		√			
31	IC515116	√				√					√
32	IC515117						√				√
33	IC525099										
34	IC538351	√						√			
35	IC538653							√			
36	IC75844	√									
37	IC548644					√		√			
38	IC568278	√				√			√		√
39	IC568250				√				√		
40	IC568228							√			
41	IC568239		√				√	√		√	√
42	IC568303		√						√		
43	IC568288		√					√		√	
44	IC568236	√								√	
45	IC568294	√					√			√	
46	IC568223						√			√	
47	IC568237						√		√	√	
48	IC568262									√	
49	RO-46		√	√		√				√	
50	JCR-1875	√								√	
51	SKY-67			√				√	√	√	
52	SKY-68	√						√		√	
53	DT 2	√		√	√	√		√	√	√	
54	DT 10										√
55	DT 13					√					
56	DT 14					√	√				
57	DT 35		√					√	√		√
58	VANDANA					√		√			√
59	SAHBHAGI									√	√
60	SADABAHAR						√				

Supplementary Fig. 2. DTY QTL survey in 60 rice genotypes

Indian J. Plant Genet. Resour. 35(2): 257–263 (2022)
DOI 10.5958/0976-1926.2022.00029.8

RESEARCH ARTICLE

SSR Marker Based Genetic Diversity and Fusarium Wilt Resistance Screening of Tomato (*Solanum lycopersicum* L.) Genotypes

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(Received: 06 April, 2022; Revised: 14 July, 2022; Accepted: 15 July, 2022)

Fusarium oxysporum is one of the devastating diseases of tomato (*Solanum lycopersicum* L.) causing high yield losses in fields and commercial greenhouses, inferring necessity for development of disease resistance. An research experiment was laid out with 23 diverse genotypes including susceptible check at research farm during wet season-2018 for screening of tomato genotypes for fusarium wilt resistance. Phenotypic screening of 23 genotypes revealed varied disease resistance as highly resistant (AVTO1219 and EC631), resistant (Pant bahar, EC620428, EC620378, EC631369 and EC620503), moderately resistant (EC615055, EC620389, EC620394, EC620422, EC620406 and AVTO9803) and moderately susceptible (PKM1, EC620382, EC620427 and EC620395) over the highly susceptible checks (Pusa Ruby, Arka Vikas). Employing 95 SSRs for molecular profiling resulted in 33 polymorphic markers, 58 monomorphic markers and remaining 4 markers as unamplified. A total of 74 alleles were detected using 33 polymorphic markers with an average allele number of 2.24 for each marker. Two markers viz., TES60 and TGS633 produced maximum (4) alleles. The polymorphic information content (PIC) value ranged from 0.28 to 0.76 with an average of 0.53 and marker TGS633 was found to be the most suitable marker with the highest PIC value. Cluster analysis through UPGMA method classified twenty three genotypes into five clusters and the coefficient among 23 genotypes was varied from 0.5 to 0.86. Over all, EC631379, EC631369, EC620428, EC620378 and EC620503 were identified as new resistant genetic resources against Fusarium wilt at both phenotypic and genotypic level with the presence of *I-2* gene loci. Based on the results, the identified genotypes can be further tested and be used in Marker Assisted Selection or gene pyramiding programs to develop disease resistant commercial cultivars.

Key Words: Cluster analysis, Fusarium wilt, Tomato: *Solanum lycopersicum*, SSRs

Introduction

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop in the world, belongs to the family *Solanaceae*, with its probable origin at Peru Equador region (Rick 1969) and it is second popular widely grown and consumed vegetable in the world, next to potato (Anonymous 2005; Reddy *et al.*, 2013). The fruits are rich source of vitamins (A and C), minerals (potassium, phosphorus, magnesium, calcium, and fair concentrations of protein and Niacin (Onyekachukwu and Adefoyeke 2017). In addition, it is also considered as 'Protective food' because of its special nutritive values and antioxidant properties due to the presence of lycopene and flavonoids (Sepat *et al.,* 2013). Lycopene is treasured for its anticancer attribute and have antiseptic and blood purifier properties. It acts as an antioxidant which is often colligated with anti-carcinogenic nature (Giovannucci 2002; Miller *et al.,* 2002; Bai and Lindhot 2007). Besides rich nutritional values, tomato also has good agronomical characteristics like wider adaptability, high yielding potential and multipurpose uses in the form of fresh and processed food Despite of the few competitors in the value addition chain of tomato, hindrances like biotic and abiotic stresses are playing detrimental role in yield formation. . Among all these hurdles, biotic stress solely effecting 10-50% of yield (de

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Carvalho *et al.,*, 2012). In tomato, over twenty diseases were reported from different parts of country and the diseases like wilt, damping-off, early blight, late blight, septoria leaf spot, leaf curl, tobacco mosaic, root-knot etc were noticed as major diseases.

Fusarium wilt (FW), caused by *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) is one of the most devastating diseases, which is a soil borne disease with mechanical mode of pathogen entry into the host and the crop is susceptible throughout all growth stages. Pathogen entry is succeeded by colonization in the xylem, resulting in inhibition of water flow and wilt like symptoms, yellowing and drooping of leaves on one side of the plant. Leaf wilting, plant stunting, browning of the vascular system, leaf death and lack of fruit production also occurs. *Fusarium oxysporum* f. sp. *lycopersici* (FOL) causes disease only in plants of the genus lycopersicon and inhabits most tomato growing regions worldwide, causing yield losses. The variation in genetic architecture of varieties will bring in them the disease resistance. Thus, the assessment of genetic diversity is essential to enhance the genetic yield potential, nutritional properties along with sustainability to different stress.

As the number of varieties continuously increase, the discrimination among cultivars based on morphological traits becomes complex as these traits are influenced by environmental factors. Molecular markers can be used as a complementary tool to overcome this and their frequent availability with the characteristics of high level of polymorphism, multi-allelic and can be assayed with minute DNA concentrations. Moreover molecular markers have also been successfully applied in registration activities, such as cultivar identification where the goal is to obtain specific pattern for each variety (Lombard *et al.,* 2001). Among different molecular markers, SSRs (microsatellites) have been most widely used over past 20 years due to its co-dominant, multi-allelic and reproducibility nature (Mason 2015). Many of the researches explored SSRs for different studies of genetic characterization and genetic assessment in tomato (Benor *et al.,* 2008; Dhaliwal *et al.,* 2011; El-Awady *et al.,* 2012; Sanghani *et al.,* 2013; Kaushal *et al.,* 2017). A combination of microsatellites can be useful in distinguishing cultivars of tomato, which are genetically strongly related to each other. The objective of this study was to evaluate tomato genotypes as new resistant sources for Fusarium wilt and assessment of genetic diversity among these tomato genotypes using molecular markers.

Materials and Methods

Plant material

A total of twenty three genotypes were collected from different sources of India. Among them 19 genotypes were procured from NBPGR, Regional Station, Hyderabad and 4 were released varieties. Details of seed material and their source were given under Table 1. The present study was carried out in wet season 2018, at the PG Research Block, Department of Vegetable Science, College of Horticulture, Sri Konda Laxman Telangana State Horticultural University, Rajendranagar, Hyderabad.

Table 1. List of genotypes and their sources used for diversity study

S. No.	Genotypes	Source
1.	EC615055	NBPGR, Hyderabad
2.	EC620463	NBPGR, Hyderabad
3.	EC620428	NBPGR, Hyderabad
4.	AVTO1219	WVC, Taiwan, China
5.	EC620378	NBPGR, Hyderabad
6.	EC620382	NBPGR, Hyderabad
7.	EC620389	NBPGR, Hyderabad
8.	EC620395	NBPGR, Hyderabad
9.	EC620406	NBPGR, Hyderabad
10.	EC620427	NBPGR, Hyderabad
11.	EC620394	NBPGR, Hyderabad
12.	EC620422	NBPGR, Hyderabad
13.	EC631369	NBPGR, Hyderabad
14.	EC631379	NBPGR, Hyderabad
15.	EC620503	NBPGR, Hyderabad
16.	AVTO9803	WVC, Taiwan, China
17.	AVTO9804	WVC, Taiwan, China
18.	AVTO1002	WVC, Taiwan, China
19.	AVTO0101	WVC, Taiwan, China
20.	Pusa Ruby	IARI, New Delhi
21.	PKM1	Periyakulam, TNAU
22.	Pant bahar	GBPUAT, Uttarakhand
23.	Arka vikas	IIHR, Bengaluru

Morphological identification and characterization of strains of FOL

For identification of Fusarium wilt causing organism strains, the morphological characteristics of micro conidia, macro conidia, phialides and chlamydospores, single-spored isolates were grown for 10-15 days on PDA medium as described by Booth (1971); Gerlach

and Nirenberg (1982); Nelson *et al.* (1983); Burgess *et al.* (1994); and Leslie and Summerell (2006). Fusarium wilt culture preparation was done followed procedure given by Nirmaladevi and Srinivas, 2012. The colour and pigmentation of the isolates on PDA medium varied between white, creamish white to cream, light pink to pink and light purple to violet.

Morphological screening

Twenty three genotypes were sown in the trays filled with coconut pith compost and raised them by following standard agronomical cultural practices. Morphological screening of 21 days old seedlings for fusarium wilt was conducted using root dip method. Conidia of all the isolates were recovered from one week old cultures. The race2 of FOL was purified and used for screening all the tomato genotypes under test. Seedlings were then removed from the trays, shaken to remove the adhering particles and washed carefully under tap water. The roots were trimmed with a sterile scissor and were submerged in the conidial suspension for 30 minutes. The inoculated seedlings were transplanted to polybags (15 cm diameter), after surface sterilized with 0.1% mercuric chloride containing soil and sand 1:1 ratio. The severity of the disease was assessed from 2 weeks of inoculation up to 45 days (Nirmaladevi and Srinivas 2012). Symptoms were recorded from 0 to 4 scale given by Bahar *et al.* (2012). 0 - No symptoms, 1- Slight chlorosis 2- Moderate chlorosis, wilting or stunting of the plant, 3- Severe chlorosis, wilting or stunting of the plant, 4- Death of the plant. In addition to this, percent incidence of Fusarium oxysporum was also calculated using a scale of 0-4 as adopted by Silme and Cagirgan (2010) which was based on infection percent as follows: 1-0.33-25%, 2-26-50%, 3-51-66.66%, 4-66.67-100%. Where: 0= Highly resistant (HR), 1= resistant (R), 2 = moderately resistant (MR), 3 = moderately susceptible (MS), 4= susceptible (S) and highly susceptible (HS).

Isolation of Genomic DNA and PCR amplification

Approximately 100 mg of fresh young leaves were collected and DNA isolation was performed by using modified CTAB method (Doyle and Doyle 1990). The quality of DNA was checked using 1% Agorase gel electrophoresis and quantity of DNA was determined with a nanodrop. These samples were diluted to the required concentration for PCR analysis and stored at -20^{o}C until use (Velpula *et al.*, 2017). PCR amplification was performed as follows: one cycle of 94^{o}C for 5 min; 35 cycles of 55^{o}C for 1 min, 72^{o}C for 2 min, and 94^{o}C for 1 min. After the final cycle, 1 cycle of 55^{o}C for 1 min and 72^{o}C for 7 min was added. The PCR amplification was verified by ethidium bromide (EtBr) were used for visualization using gel documentation unit.

SSR and Cluster analysis

A total of 95 SSRs were used to analyse the genetic diversity of 23 tomato genotypes, which were selected from the linkage map of tomato genome by covering all the 12 chromosomes. The amplified alleles for 23 genotypes with each primer were scored based on presence or absence of the allele at a given locuswith1 and 0 respectively. The polymorphism information content (PIC) for each SSR was calculated according to the formula PIC = 1 - $\sum pi^2$ .Where pi is the frequency of the i^{th} allele for each SSR marker locus in the set of 23 tomato genotypes investigated (Weir 1990). The binary matrix data retrieved from the molecular marker analysis was used for diversity assessment. The Jaccard's similarity coefficient values were calculated for 23 tomato germplasm accessions using NTSYS-pc version 2.02e (Rohlf 2000) and the dendrogram was constructed based on UPGMA (Unweighted pair group method with arithmetic mean) method.

Results

Morphological screening

In the present study, 23 genotypes were studied for Fusarium wilt resistance at morphological level. Scoring of Fusarium wilt infection severity based on morphological symptoms revealed five groups (Table 2) *viz.,* asymptomatic/no chlorosis in two cultivars (AVTO1219 and EC631) (Supplementary Fig. 1), slight chlorosis of leaves in five genotypes (Pant bahar, EC620428, EC620378, EC631369 and EC620503), Moderate chlorosis with wilting or stunting of the plant in six genotypes (EC615055, EC620389, EC620394, EC620422, EC620406 and AVTO9803), severe chlorosis with wilting and stunting of the plant in four genotypes (PKM1, EC620382, EC620427 and EC620395) and plant death was observed in six genotypes (EC620463, AVTO9804, AVTO1002 and AVTO0101) including susceptible checks (Pusa Ruby and Arka Vikas). The percent incidence of disease in genotypes was ranged from 0% to 100% (Supplementary Table 1).

Table 2. Response of genotypes for *Fusarium* wilt incidence

S. No.	Reaction	Score	Number of genotypes	Genotypes
1.	Highly Resistant (HR)	0	2	AVTO1219, EC631379
2.	Resistant (R)	1 (0-1)	5	Pant bahar, EC620428, EC620378, EC631369, EC620503
3.	Moderately Resistant (MR)	2 (1-2)	6	EC615055, EC620389, EC620394, EC620422, EC620406, AVTO9803
4.	Moderately Susceptible (MS)	3 (2-3)	4	PKM1, EC620382, EC620427, EC620395
5.	Susceptible (S) and Highly Susceptible (HS)	4 (3-4)	6	Pusa Ruby, Arka Vikas, EC620463, AVTO9804, AVTO1002, AVTO0101

Molecular screening

The PCR analysis using 95 SSRs resulted 33 markers as polymorphic (Fig. 3), 58 markers as monomorphic and remaining 4 markers were not amplified. These 33 polymorphic markers were further used in analysis of molecular diversity with yielded allelic data. The binary matrix data was prepared by considering clear variation among alleles. The total number of alleles were 74 with an average of 2.24 and the the number of alleles were varied from 2 to 4. Highest number of alleles was observed with TES60 and TGS633. PIC value among SSRs was varied widely from 0.28 to 0.76 with an average of 0.53 (Supplementary Table 2). Highest PIC value of 0.76 was observed with TGS633 and SSR46, TGS1093 were followed with 0.73 PIC value. Whereas the lowest PIC value of 0.28 was observed with TEI0396 marker.

Genetic diversity pattern

Based on UPGMA cluster analysis using 33 polymorphic primers, the 23 genotypes were classified into 5 clusters *i.e.* cluster I, cluster II, cluster III, cluster IV and cluster V with the similarity coefficient value from 0.5 to 0.85 (Supplementary Fig. 2). List of genotypes in clusters I to V were given under Supplementary Table 3. The highest similarity was observed between EC620382 and EC620389, placed in sub cluster II with 86% similarity.

Discussion

A total of 23 tomato genotypes were screened for Fusarium wilt resistance after proper confirmation of fungal isolates (*Fusarium oxysporium)* symptoms. Interestingly, different levels of resistance was observed among the genotypes (Table 2), indicating high diversity among the genotypes. The two genotypes AVTO1219, EC631379 with 0% disease incidence and with score of "0" were showing their high resistance nature against Fusarium wilt, this resistance nature of AVTO1219 was also explained by World Vegetable Centre database, Loganathan Indian Institute of Spices Research annual report (2012-13). Genotypes, Pusa Ruby, EC620463, AVTO1002, Arka Vikas, AVTO0101 and AVTO9804 were susceptible genotypes with disease incidence of 100-83.33 (3-4 score). In the present study, genotypes with varied disease symptoms (scored as 0-4) and disease incidence (80% -100% incidence) were observed. These results are in agreement with the finding of Mahmoud *et al*. (2006); Ahmadvand *et al.* (2010) and Antonio *et al.* (2017).

In addition to this, genetic diversity among 23 genotypes was assessed using 95 SSR's markers, which were selected from the linkage map of tomato genome by covering all the 12 chromosomes. The PCR analysis using 95 SSRs revealed 33 polymorphic markers, 58 monomorphic markers and 4 unamplified markers. Thus, these markers showed low levels of polymorphism among genotypes (Alvarez *et al.,* 2001; Yang *et al.,* 2005), it may be due to the non specificity of markers and the lower levels of polymorphism was detected by interrupted and imperfect SSRs may be associated with the initial stages of mutational decay, so that replication slippage is less-likely to occur (Smulders *et al.,* 1997; Benor *et al.,* 2008) or probably due to its autogamous nature (El-Awady *et al.,* 2012). A total of 74 alleles were observed with 33 polymorphic markers with an average of 2.24 alleles for each marker. The highest number of alleles (3) was resulted with markers TES60 and TGS633 showing the importance of these markers in the diversity analysis. PIC value was calculated based on allelic information to study the discrimination power of each marker among the genotypes. As a result it was in the range of 0.28 to 0.76 with an average value of 0.53. This results was almost similar to Jones *et al.*, (2001) in the range of 0.2 and 0.8 with an average value

0.56 and it was higher than the previously reported PIC values of different diversity studies conducted in tomato like 0.3 (Kaur *et al.,* 2018), 0.31 (Benor *et al.,* 2008), 0.35 (Varshney *et al.,* 2009), 0.39 (Frary *et al.,* 2005), 0.37 (He *et al.,* 2003), 0.4 (Bredemeijer *et al.,* 2002), 0.45 (Glogovac *et al.,* 2013),. Interestingly, highest PIC value of 0.76 was observed with TGS633 and SSR46, TGS1093 were followed with 0.73 PIC value indicating these markers would be further useful to discriminate the genetic variability in tomato germplasm. Moreover the marker TGS633 was making remarkable role with highest allele value along with highest PIC value.

Based on cluster analysis using UPGMA was revealed all the genotypes into five major clusters (I, II, III, IV and V). Similarity coefficient among 23 genotypes was observed with the range of 0.5 to 0.86. The superior nature of Pusa Ruby was once again proved by forming into a separate cluster (I) among all genotypes (Yogendra and Gowda 2013). In second cluster the two genotypes EC620382 and EC620427 were shown highest similarity (86%), this highest similarity is may be due to the similarities in evolution pattern. Interestingly, these two genotypes showed negligible (0.81) similarity coefficient, though from different pedigree. The remaining two released varieties PKM1 and Pant Bahar were also located in the same clustered and paired with AVTO1219 with different levels of resistance against Fusarium wilt. Most of the remaining lines in cluster II were shown similar results at both genotypic and phenotypic level except EC631379 and Pant bahar, it may be due to the presence of other resistance gene loci of Fusarium wilt and the resistant of Pant bahar was also explained by Agarwal *et al.* (2000). In cluster III, all the genotypes were followed the same pattern in both the cases (phenotypic and genotypic). As expected, all the AVTO genotypes were placed in cluster IV along with EC620463 except AVTO1219. Even though EC620463 was located in sub cluster B2, it was showing the superiority over the other genotypes of cluster IV. In addition to this, AVTO1219 was placed very nearer to the Pant bahar and PKM1. Similarly in cluster IV all the genotypes were phenotypically susceptible to FW except AVTO9803 and it was resulted same with the use of FW gene specific primer. Whereas the genotype EC620406 classified as a cluster V was not identified with any allele with FW specific primer but it has shown moderately resistance at phonotypical screening. Interestingly all the AVTO genotypes were aligned as one group and all the EC lines were aligned in another group with three released varieties and AVTO1219. Whereas, the two genotypes including one released variety (Pusa Ruby) and one Exotic collection (EC620406) were found to be most diversifying among all the genotypes used in this study by truncated into separate clusters with lowest similarity. Over all, EC631379, EC631369, EC620428, EC620378 and EC620503 were identified as new resistant genetic resources against Fusarium wilt at both phenotypic and genotypic level with the presence of *I-2* gene loci. Thus, the identified highly diversified/ related tomato genotypes can be improved further using various breeding and crop improvement programmes.

Conclusion

In the present study a total of 23 genotypes were screened for Fusarium wilt resistance. The morphological screening was revealed EC631379 and AVTO1219 as highly resistant genotypes. Some more genotypes *i.e.,* Pant bahar, EC620428, EC620378, EC631369 and EC620503 were also resulted as resistance against Fusarium wilt at phenotypic level. Remarkably this resistance of some EC lines *viz*., EC615055, EC620428, EC620378, EC620389, EC620394, EC620422, EC631369 and EC620503 were also confirmed genetically using Fusarium wilt resistance I-2 gene specific primer along with AVTO1219 and AVTO9803. In addition to this the genetic assessment using SSRs revealed primer TGS633 as most useful marker in tomato genetic diversity and using the cluster analysis. Pusa Ruby and EC620406 were resulted as high diversified genotypes among 23 genotypes using SSRs. Over all, the new genetic resources were identified for resistance to Fusarium wilt race2 at phenotypic and genotypic level. Hence, the genotypes identified with the presence of I-2 gene can be further useful for marker assisted breeding program for the improvement of tomato crop.

Acknowledgement

Authors are thankful to SKLTSHU, Rajendranagar, Hyderabad for providing facilities to carry out the research trial.

*Supplementary Table or Figure mentioned in the article are available in the online version.

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Supplementary Table 1. Scores and percent incidence of fusarium wilt in 23 genotypes

S.No.	Genotype	Score	Percent incidence	S.No.	Genotype	Score	Percent incidence
1.	Pusa Ruby	4.00	100.00	14.	EC620427	2.33	58.33
2.	PKM1	2.33	58.33	15.	EC620394	2.00	50.00
3.	Pant bahar	0.67	16.66	16.	EC620422	1.67	41.66
4.	Arkavikas	3.33	83.33	17.	EC631369	1.00	25.00
5.	EC615055	1.33	33.33	18.	EC631379	0.00	0.00
6.	EC620463	4.00	100.00	19.	EC620503	0.67	16.66
7.	EC620428	0.33	8.33	20.	AVTO9803	2.00	50.00
8.	AVTO1219	0.00	0.00	21.	AVTO9804	3.33	83.33
9.	EC620378	0.33	8.33	22.	AVTO1002	3.67	91.66
10.	EC620382	2.33	58.33	23.	AVTO0101	3.33	83.33
11.	EC620389	1.67	41.66		C.V	8.32	14.89
12.	EC620395	2.67	66.66		C.D (P=0.05)	2.13	9.86
13.	EC620406	1.33	33.33				

Supplementary Table 2. List of polymorphic primers with sequence, Chromosome number, Annealing temperature, Number of alleles and PIC

S.No.	Primer	Sequence 5^1 to 3^1	Chromosome number	Alleles	**PIC**
1	TGS2458	F:GTGAATTTTTCAAACCCTGGC	1	2	0.58
		R: ATTTGGAAATGAACTCGGCA			
2	TES109	F:GTCAACAACTATTCCAGGCCC	1	2	0.53
		R: CTCCCGTGCAAAATCTAAGC			
3	SSR222	F: TCTCATCTGGTGCTGCTGTT	1	2	0.58
		R: TTCTTGGAGGACCCAGAAAC			
4	TES134	F: GTCATTTTTCCCCAGCTGTTC	1	2	0.53
		R: AAGGAAAAGACCCAGGTGTG			
5	SSR37	F: ATTGAAGACCGAAACGGTTG	1	2	0.53
		R: CTGATAAACCCGGCAAGACT			
6	SSR32	F: TGGAAAGAAGCAGTAGCATTG	2	2	0.53
		R: CAACGAACATCCTCCGTTCT			
7	SSR22	F:GATCGGCAGTAGGTGCTCTC	3	2	0.62
		R:CAAGAAACACCCATATCCGC			
8	SSR3	F:CTAATATAGTAGAGTAGGAGTAAG	3	2	0.53
		R:GCTCTAATGATAAGGAGAGAGTCTG			
9	TES60	F:GTTCCTCCTCCTCCTCCTTTC	4	4	0.45
		R: ACACAATTCCCCAAAATCCA			
10	SSR214	F:AAATTCCCAACACTTGCCAC	4	2	0.53
		R:CCCACCACTATCCAAACCC			
11	TES1652	F:AAAAAGTCAGCTTCAGTGGTAGTATAG	4	3	0.30
		R:GCAACCTCCTACTCTGCTGG			
12	TGS633	F: GTTTTTACCAATTCTTCCGGC	5	4	0.76
		R:AGGAAATAGAATAAAACTAACCCAAA			
13	TGS914	F: GCCAGGCATTCCAACAATACA	5	2	0.61
		R: TCACTTGTGCAATGAGGTTGA			
14	SSR162	F: GCTCTCTACAAGTGGAACTTTCTC	5	2	0.50
		R: CAACAGCCAGGAACAAGGAT			
15	TES1743	F: GAGTGTCTCGATCTCGCACCT	6	2	0.53
		R: CCATGTGTCCAACCTTTTCC			

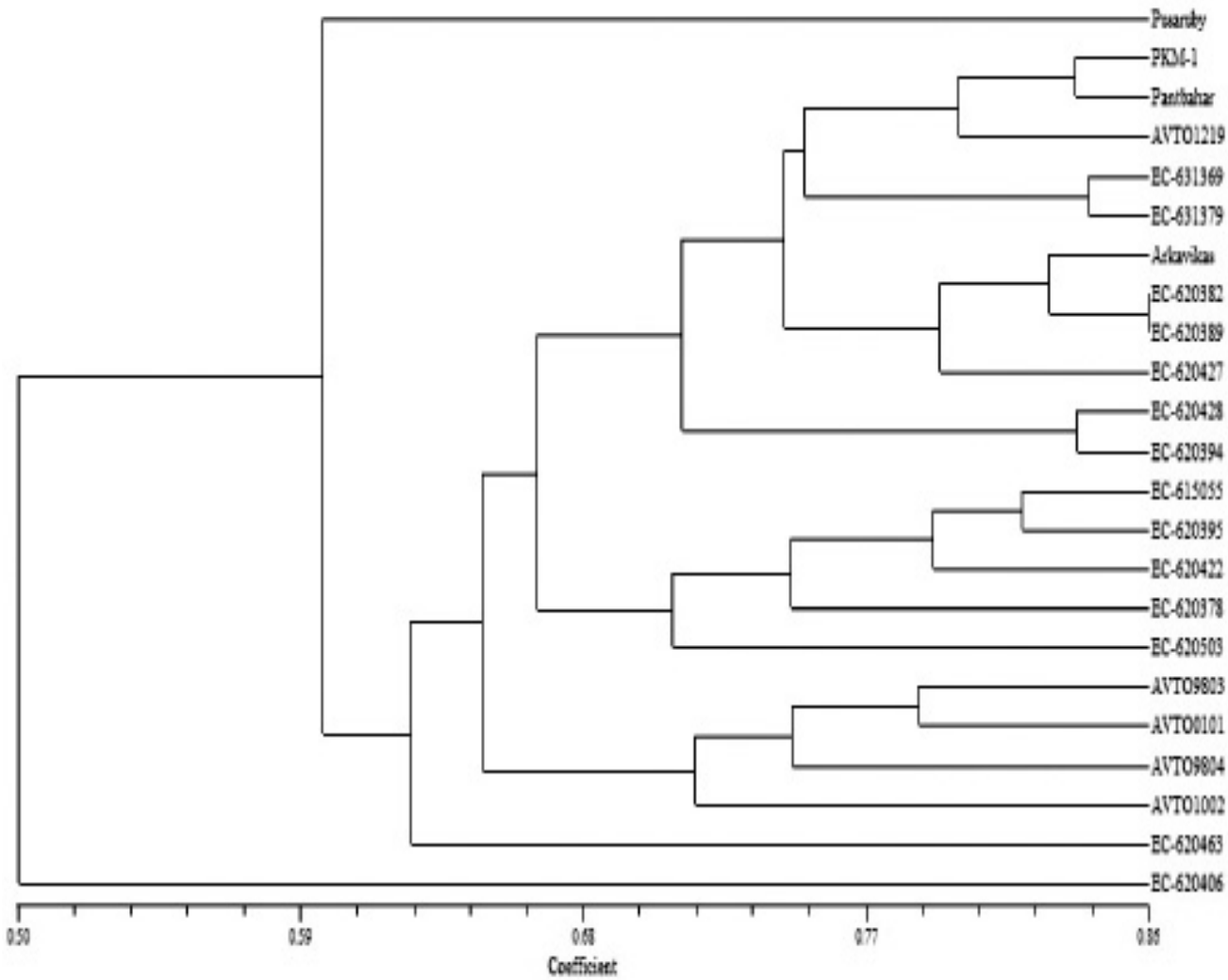
S.No.	Primer	Sequence 5[1] to 3[1]	Chromosome number	Alleles	**PIC**
16	TGS2005	F: GGGTGAAAGGATAAGGGAAA	7	2	0.58
		R: CGGATTTCTTGTTGTGTTGC			
17	TGS192	F: GTCAGTTGCTTTTTATCCAACAA	7	2	0.36
		R: CACTGATGGGAATGCCTTTT			
18	SSR8-0.5	F:GTAATCTTACTTTAGATGACATG	8	2	0.58
		R:CCATAAGAATACAATCCACTTG			
19	TGS610	F: GTTAGTGAAGTGAAGAGGAAGCAA	8	3	0.42
		R: CCGGCAAGCTGCATTTTT			
20	TES36	F: GGACCAAGCGAAGTTGGATA	9	2	0.61
		R: CGAGTGTTTCGCTTCTCCTC			
21	TES184	F: GCGTCATCAACCAGTCAGCAG	9	2	0.64
		R: TATTTCTGTGCCAATGGACG			
22	SSR112	F: GGAACACAACCAAGAAGTGGA	9	2	0.35
		R: TATCGGCTTAGGGTTGTTGG			
23	TES1592	F: GCCAATTTGGTGGCTACCCT	9	3	0.65
		R: CGGGATATCTGCCTCTACCA			
24	TES1154	F: GAGCGACCTCAACTTGTTTGG	10	2	0.49
		R: AACCAGATGACCCCATTTGA			
25	TGS643	F: GTTTCTCCCAAGGGGGATATT	10	3	0.53
		R: ACTTCCAAGCGGGGATAGAT			
26	TEI0396	F: GCTATGTATAGGAAGCAACACAAGA	10	2	0.28
		R:TAGCAGCTTCTTGGGCGATA			
27	SSR223	F: TGGCTGCCTCTTCTCTGTTT	10	2	0.53
		R: TTTCTTGAAGGGTCTTTCCC			
28	TES0426	F: TTTGAGGAGGGCTGAAGAGA	11	2	0.58
		R: GCAGGATAACAGCCTCTTGC			
29	SSR46	F: CCGAGGCGAATCTTGAATAC	11	2	0.73
		R: GCACCATCTCTTGTGCCTCT			
30	TES152	F: GTGTTTCTATTCGTGAACCATGA	11	2	0.65
		R: CCGTGAGTTAGCTAATGAGGTT			
31	TGS1476	F: GTCATGGGAATGACACTAACGAG	11	2	0.29
		R: AGTGTGTGTGTTTGTGTGCG			
32	TES1420	F: GCAGCTCGTCATTTCTTCAA	12	2	0.61
		R: AGTGGCTGAAGAAGAACGGAA			
33	TGS1093	F: GTTTCTTCTTTGTAAATCGGCG	12	2	0.73
		R: CGAGTCAACCCCTAGGCTAC			

PIC: Polymorphic information content

Supplementary Table 3. Clustering pattern of genotypes obtained by genetic diversity analysis

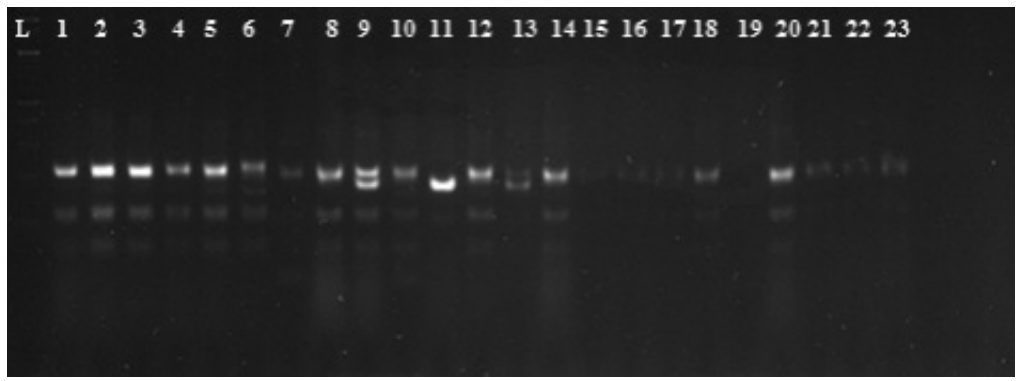
Cluster Number	Number of genotypes	**Name of genotypes**
I	1	Pusa Ruby
II	11	PKM1, Pant bahar, AVTO1219, EC631369, EC631379, Arka Vikas, EC620382, EC620389, EC620427, EC620428, EC620394
III	5	EC615055, EC620395, EC620422, EC620378, EC620503
IV	5	AVTO9803, AVTO0101, AVTO9804, AVTO1002, EC620463
V	1	EC620406

Supplementary Fig. 1. Disease reaction in genotypes AVTO1219, EC631379 (Resistant), Pusa Ruby (Susceptible check)



Supplementary Fig. 2. Dendrogram obtained from SSRs analysis using UPGMA analysis based on 33 SSR primers Banding pattern of SSR markers

TGS610



TES109

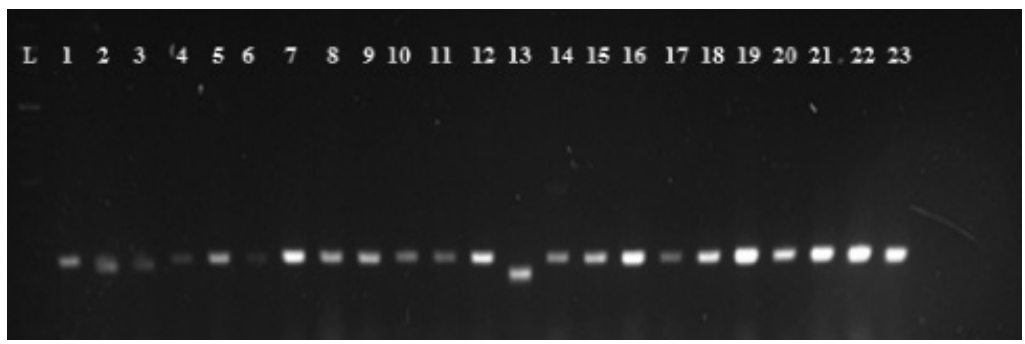


Fig. 3. Banding pattern of SSR markers

Indian J. Plant Genet. Resour. 35(2): 264–274 (2022)
DOI 10.5958/0976-1926.2022.00030.4

RESEARCH ARTICLE

Improved Micropropagation Protocol and Molecular Marker Based Genetic Stability Assessment of Black Pepper (*Piper nigrum* L.)

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(Received: 21 June, 2022; Revised: 24 July, 2022; Accepted: 25 July, 2022)

Black pepper (*Piper nigrum* L.) is a highly commercial and valuable spice crop in the world, plants of which have originated from Western Ghats region of southern India. An efficient and reliable micropropagation protocol is reported in this paper, which can be utilized for multiplication of elite genotypes of black pepper. Shoot regeneration was induced on Murashige and Skoog (MS) and Woody Plant Media (WPM) media in 24 combinations with plant growth regulators. Explants cultured in MS media supplemented with 0.5 mg L^{-1} BAP formed 6.5 nodes per plant with longest shoots (10.42 cm) after 12 wks. Regenerated shoots were rooted on MS media supplemented with 2.0 mg L^{-1} IBA, yielding about 15 roots per plant with an average root length of 3.40 cm within 4 wks. The hardening of the shoots under a mist chamber conditions (25±2 °C, 70-80% RH) yielded 100% survival after acclimatization. Genetic stability analysis of the micropropagated and mother plants was done using 48 ISSR markers. The plants found to be genetically stable when compared with mother plants. A ratio of 1:6.5 numbers of nodes is obtained on the MS medium supplemented with 0.5 mgL^{-1} of BAP within 3 months. This research presents an improved and efficient micropropagation protocol with good rate of multiplication of the genetically stable plants that are easily acclimatized to the field conditions.

Key Words: ***In vitro* clonal propagation, *Piper nigrum*, Black pepper, Molecular markers, Field acclimatization**

Introduction

Black pepper (*Piper nigrum* L., Family Piperaceae) is one of the world's most important and commonly used spice. Due to its enormous usage, volume of trade and commerce among spices in the world market, the crop is known as the "king of spices" (Srinivasan, 2007). It is found growing in a wide range of altitudes and has been shown to be tolerant to a variety of environmental conditions (Ravindran and Kallupackaral, 2012). Due to the color of the peppercorn seed, the economically important portion of black pepper, it was given the name "black pepper". It is native to India, particularly the Western Ghats region. Vietnam is the world leader in terms of total production (0.267 million tons). It is grown on 0.259 million ha in India, with a production of 61 thousand tons, of which 16,250 tons were exported, earning INR 5.51 billion worth in foreign currency (Spice Board of India, 2020). Karnataka is the major producer of black pepper in India.

Black pepper is a woody climbing vine, with aerial roots trailed over the support of columns and can grow up to a height of 5-6 m. It features bright, lustrous leaves that are placed alternately. The fruits, which are drupes (~5 mm dia), are the economically important part. Black pepper is a mature fruit that has been dried (peppercorns). Pepper is used in a multitude of ways, mostly as a spice, a condiment, a preservative, an insecticide and as herbal medicine (Wang *et al*., 2017). The component piperine, a pungent alkaloid, is responsible for the black pepper's spicy flavor (De Almeida, 2020). It improves the bioavailability of a wide range of other structural and therapeutic medicines (Khajuria, 2002). It is used in a diverse variety of cuisines around the world.

Piper nigrum plant is a climber, propagated *via* seeds, cuttings (2-6 nodes per cutting), layering and grafting. Due to the heterozygous nature of the seeds, propagation *via* cuttings is commonly used and other techniques of propagation are not used because they are slow and time demanding (Ramakrishna Nair and Datta Gupta, 2003; Hussain *et al*., 2011). Vegetative cultivation of black pepper is hindered by diseases such as foot rot and anthracnose and also insect such as pollu beetle

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(*Longitarsus nigripennis)*. Cucumber mosaic virus and piper yellow mosaic virus are examples of viruses that can be transmitted to progenies (Bhat *et al*., 2018).

The germplasm of black pepper that is free of pest and diseases can be easily maintained and mass produced using plant tissue culture techniques. The exploitation of *in vitro* culture techniques has allowed researchers the unique opportunity to micropropagate plants from somatic tissues, overcoming sexual barrier (Abbasi *et al*., 2010). This method results in disease-free, pest-free and virus-free plants, which are genetically stable and produce identical progenies. Although, the black pepper crop can be grown in tissue culture, there are just a few reports of it being propagated using large-scale micropropagation technique. Explants employed include callus, somatic embryos (Ramakrishna Nair and Dutta Gupta, 2003), shoot tips (Nazeem *et al*., 1992; Philip *et al*., 1992; Babu *et al*., 1993; Joseph *et al*., 1996), nodal explants (Bhat *et al*., 1995) and leaf explants (Sujatha *et al*., 2003). The existence of endogenous bacterial infection is known to impair establishment of fresh *in vitro* cultures (Fitchet, 1990; Philip *et al*., 1992; Abbasi *et al*., 2010).

By analyzing the importance of black pepper crop and the lack of sufficient reports on efficient direct shoot generation and shoot multiplication protocol, in the present study, 24 permutations and combinations of media which included two types of basal media and two types of plant growth regulators were tested for micropropagation protocol development. Further, regenerated plants were tested for genetic stability using molecular markers.

Materials and Methods

Plant material and culture conditions

Piper nigrum accession TCR59 (IC 85371), maintained in the *In Vitro* Gene Bank (IVGB) of ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India, was utilized as mother culture (free from endogenous bacteria) to establish fresh cultures. Nodal segments (1-1.5 cm) were excised from 4-wks-old cultures and were further used as explants in the experiments (Fig. 4A).

The experimental media consisted of two basal media combinations *viz*., Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and Woody Plant Medium (WPM) supplemented with 3% (w/v) sucrose and agar (0.8%) (Himedia Laboratories, Mumbai, India) and plant growth regulators (PGRs) (Sigma- Aldrich, Saint Louis, MO, USA) as shown in Table 1. The pH of media was adjusted at 5.8 using 1N NaOH and HCl prior to autoclaving (121°C at 15 psi pressure for 17 min). All the cultures were incubated under a standard culture room conditions maintaining 25±2°C, 16/8 h photoperiod and a light intensity of 40 $\mu Em^{-2} s^{-1}$ using white fluorescent light (Philips, India).

Shoot proliferation and elongation

Experiments were undertaken to evaluate the shoot regeneration and elongation on MS and WPM media supplemented with 24 permutations and combinations of PGRs comprising kinetin (Kn) and 6-BenzylAmino-Purine (BAP) ranging from 0.25 mg L^{-1} to 2.5 mg L^{-1} (Table 1). Approximately 20 ml of the medium was dispensed into culture tubes (25×150 mm; Borosil, Mumbai, India). Data on shoot length (cm), number of nodes and number of leaves per plantlet were recorded biweekly, up to 12 wks. Based on the data on number of nodes per plantlet and shoot length obtained after 12 wks of culturing, the best medium for shoot proliferation and shoot elongation was identified.

In vitro rooting

For root induction, well-developed and healthy *in vitro* grown shoots (with 3 nodes) were transferred to MS basal salts (3% sucrose, 0.8% agar) supplemented with different concentrations of Indole-3-Butyric Acid (IBA) ranging from 0.5 mg L^{-1} to 2.0 mg L^{-1} and full- and half-strength MS medium (Table 2). Approximately 20 ml of the medium was dispensed into culture tubes (25×150 mm; Borosil, Mumbai, India). Data on number of shoots forming root, root length (cm) and number of roots per shoot were recorded after four wks. Average root number and root length per shoot were computed.

Acclimatization to ex vitro conditions

Plantlets with well-developed roots with approximately 4-5 nodes measuring 7-9 cm were removed from culture tubes and rinsed under running tap water to remove any adhering medium. The washed plantlets were planted separately in protrays filled with a mixture of autoclaved horticultural grade perlite: Irish peat moss mixture in the ratio of 25:75 (Glasil Scientific, New Delhi, India). The plantlets were watered with MS basal salt solution during first week. The tray was covered with transparent polythene bags in order to maintain the high relative humidity (70-80 %) in a mist chamber under normal

Table 1. Effects of different concentrations of plant growth regulators on shoot growth of black pepper (*Piper nigrum* L.) on Murashige and Skoog (MS) medium and Woody Plant Medium (WPM)

Serial no.	Basal medium	Medium code	Plant growth regulator (mg L^{-1})		Shoot length (cm)	Number of nodes/shoot	Number of leaves/ shoot	Callus response
			Kn	BAP				
1	MS	K1	0.25	-	6.60 ± 0.25^{defg}	3.58 ± 0.37^{cde}	3.66 ± 0.14^{efgh}	
2		K2	0.5	-	7.43 ± 0.36^{cde}	3.25 ± 0.25^{cdef}	3.75 ± 0.13^{defg}	
3		K3	1.0	-	8.32 ± 0.44^{bcd}	2.33 ± 0.14^{efg}	3.25 ± 0.13^{ghij}	+
4		K4	1.5	-	8.42 ± 0.66^{bc}	2.58 ± 0.28^{efg}	3.08 ± 0.31^{ghij}	+
5		K5	2.0	-	9.45 ± 0.35^{ab}	2.75 ± 0.21^{defg}	3.50 ± 0.19^{fghi}	+
6		K6	2.5	-	7.78 ± 0.60^{bcde}	6.08 ± 0.89^{b}	3.58 ± 0.19^{efghi}	+
7		B1	-	0.25	10.29 ± 0.94^{a}	4.08 ± 0.48^{cd}	4.58 ± 0.41^{bcd}	+++
8		**B2**	**-**	**0.5**	$\mathbf{10.42 \pm 0.78^{a}}$	$\mathbf{6.58 \pm 0.65^{b}}$	$\mathbf{5.58 \pm 0.35^{a}}$	+++
9		B3	-	1.0	8.30 ± 0.64^{bcd}	5.83 ± 0.50^{b}	4.41 ± 0.28^{bcde}	+++
10		B4	-	1.5	6.31 ± 1.25^{efg}	4.25 ± 1.03^{c}	3.66 ± 0.67^{efgh}	+++
11		B5	-	2.0	8.26 ± 0.63^{bcd}	8.33 ± 0.80^{a}	5.25 ± 0.27^{ab}	+++
12		B6	-	2.5	2.63 ± 0.72^{g}	1.66 ± 0.78^{g}	1.75 ± 0.53^{k}	+++
13	WPM	WK1	0.25	-	7.03 ± 0.24^{cdef}	2.16 ± 0.11^{efg}	3.16 ± 0.11^{ghij}	+
14		WK2	0.5	-	6.90 ± 0.17^{cdefg}	2.16 ± 0.11^{efg}	2.91 ± 0.08^{ghij}	+
15		WK3	1.0	-	6.83 ± 0.36^{cdefg}	2.00 ± 0.00^{fg}	2.75 ± 0.13^{hij}	++
16		WK4	1.5	-	6.76 ± 0.29^{cdefg}	1.83 ± 0.11^{fg}	2.50 ± 0.15^{jk}	++
17		WK5	2.0	-	6.62 ± 0.27^{cdefg}	2.16 ± 0.16^{efg}	2.66 ± 0.14^{ij}	++
18		WK6	2.5	-	6.90 ± 0.17^{cdefg}	2.16 ± 0.16^{efg}	2.91 ± 0.14^{ghij}	++
19		WB1	-	0.25	6.78 ± 0.26^{cdefg}	4.16 ± 0.27^{cd}	4.66 ± 0.18^{bc}	++
20		WB2	-	0.5	6.90 ± 0.21^{cdefg}	4.08 ± 0.19^{cd}	4.83 ± 0.11^{abc}	++
21		WB3	-	1.0	6.92 ± 0.21^{cdefg}	4.41 ± 0.19^{c}	4.83 ± 0.24^{abc}	++
22		WB4	-	1.5	5.19 ± 0.34^{g}	4.25 ± 0.44^{c}	4.25 ± 0.35^{cdef}	+++
23		WB5	-	2.0	6.05 ± 0.23^{efg}	4.50 ± 0.15^{c}	4.83 ± 0.16^{abc}	+++
24		WB6	-	2.5	5.63 ± 0.46^{fg}	4.25 ± 0.25^{c}	4.33 ± 0.30^{cdef}	++

Means ± SE within a column superscripted by the same letter are not significantly different at P≤ 0.05
BAP: 6-benzylaminopurine; Kn: kinetin

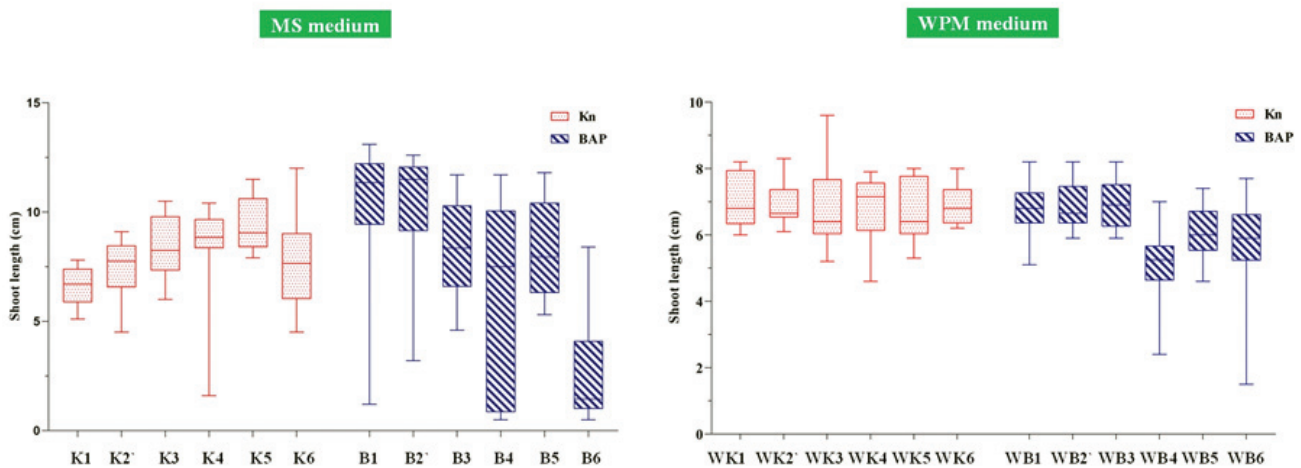

Fig. 1. Box plot representation of shoot length in cultures of *Piper nigrum* L. in MS and WPM media supplemented with Kn and BAP (for media codes refer to Table 1).

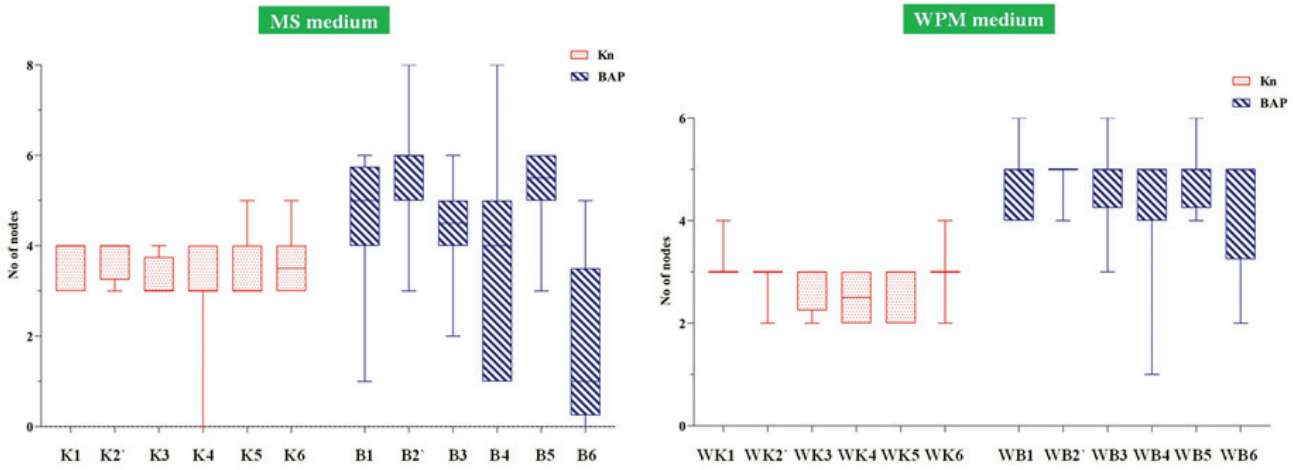

Fig. 2. Box plot representation of number of nodes in cultures of *Piper nigrum* L. in MS and WPM media supplemented with Kn and BAP (for media codes refer to Table 1).

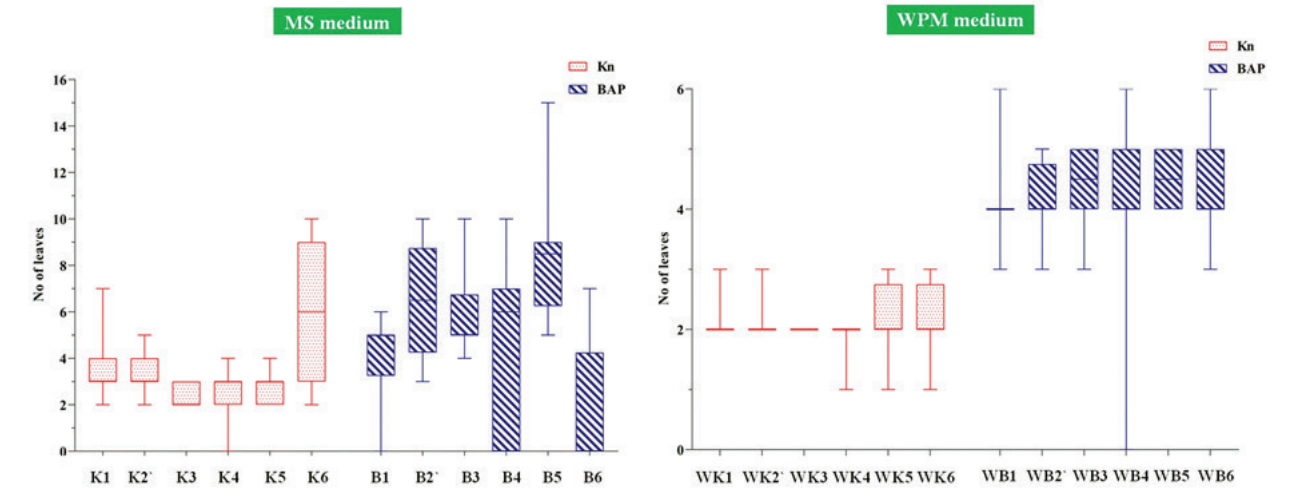

Fig. 3. Box plot representation of number of leaves in cultures of *Piper nigrum* in MS and WPM media supplemented with Kn and BAP (for media codes refer to Table 1).

growth conditions (16/8 h of photoperiod, 25±2°C). After 2-3 days small holes (4-5) were made for aeration in the polythene bags. After first week, once in every five days plants were irrigated using Hoagland solution (HiMedia laboratories, Mumbai, India). The seedlings (hardened plants) were transferred to earthen pots (10'' dia) with soil and farmyard manure (1:1) after 4 wks and kept under the shade net conditions. The survival of the rooted plants was documented after 3 wks of hardening.

Statistical analysis

All the experiments were carried out in 12 replications by taking one culture as one replicate and repeated twice using completely randomized design (CRD). Data on shoot length, number of nodes, leaves and root, rooting percentage and root length (cm) are represented as mean ± standard error. Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used for comparison and for significant difference among means (Duncan, 1955) ($P \leq 0.05$) using SPSS statistics version 22.0 software package. Two-way ANOVA data were subjected to post-hoc Sidak's multiple comparison test ($P \leq 0.05$) utilizing GraphPad Prism 9.1.0 software.

Assessment of genetic stability

Genetic stability analysis was carried out by comparing mother stock cultures (12 replicates) with regenerants from the two best micropropagation media, namely K2

Fig. 4. *In vitro* propagation of *Piper nigrum* L. using nodal segment as explant: (A) Nodal segment explants (B) Shoot development on MS media supplemented with 0.5 mg/L of BAP (C) Root induction on MS media supplemented with different doses of IBA (0.5-2.0 mg L^{-1}), full-and half-strength MS medium (D) Rooted plant before transplanting into seedling trays (E) Plants 3 week after transplanting (F) Plants transplanted to pots

(MS+ 0.5 mg L^{-1} Kn) and B2 (MS + 0.5 mg L^{-1} BAP). The DNA from young leaves (from 3-month-old culture) were isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987) with slight modifications. The quality and the quantity of DNA were estimated using Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

A total of 48 ISSR primers (Eurofins Genomics India Pvt. Ltd., Bengaluru, India) were tested for PCR amplification (Table 4). The reaction mixture comprised DNA template (3 µl), 5 µl master mixture (1x), 0.5 µl primer and 1.5 µl water in 10 µl reaction volumes. Primers yielding reproducible bands were used for the analysis. Reaction was carried out in a thermal cycler (Gene Pro, Hangzhou Bioer Technology Co., Hangzhou, China). The thermal cycler was programmed for (i) Initial denaturation (94°C for 2 min); (ii) Denaturation (94°C for 10 sec); (iii) Primer annealing temperature (T_m) based on primer for 30 sec (iv) Primer extension (72°C for 65 sec); (v) Final extension (72°C for 10 min) and 4°C as holding temperature. The amplified PCR products were stored at 4°C for further analysis.

The ISSR-PCR amplification products were analyzed by gel electrophoresis in 2% agarose gel immersed in 1xTAE buffer (G-Biosciences, Saint Louis, MO) which stained with Ethidium Bromide (Etbr) and was run at a constant voltage of 5 V cm^{-1}. All the amplified products were electrophoresed and gel was imaged by Gel Documentation System (GenoSens 2100, Clinx Science Instruments Co., Shanghai, China). The banding pattern of the mother plant and the micropropagated plant were compared to record for any variation existing at the molecular level.

Results and Discussion

Shoot proliferation

An optimal selection process for an efficient micropropagation protocol involves the selection of a suitable explant, as well as the proper combinations of the PGRs in optimum ratio, as these factors have a direct impact on the shoot and root proliferation of the plantlets. In this study, various characters like the shoot length, number of nodes and number of leaves were influenced by two types of the basal media (MS and WPM) and to varying concentrations of the Kn and BAP tested.

In the present study, among the 24 combinations of cytokinins tested, BAP alone in MS medium was found better with respect to shoot growth and multiplication (Table 1). On MS medium mean shoot length ranged from 2.63 cm (2.5 mg L^{-1} BAP) to 10.42 cm (0.25 mg L^{-1} BAP) (Fig. 1a); number of nodes ranged from 1.66 (2.5 mg L^{-1} BAP) to 8.33 (2.0 mg L^{-1} BAP) (Fig. 2a); leaves from 1.75 (2.5 mg L^{-1} BAP) to 5.58 (0.5 mg L^{-1} BAP) (Fig. 3a). Contrarily on WPM media, the average shoot length ranged from 5.19 cm (1.5 mg L^{-1} BAP) to 7.03 cm (0.25 mg L^{-1} Kn) (Fig. 1b); number of nodes ranged from 1.83 (1.5 mg L^{-1} Kn) to 4.50 (2.0 mg L^{-1} BAP) (Fig. 2b); number of leaves ranged from 2.50 (1.5 mg L^{-1} Kn)to 4.83 (0.5 mg L^{-1} BAP) (Fig. 3b). A cream colored hard nodular callus formed at the cut end of the explants, which did proliferate significantly (Table 1). ANOVA analysis of the micropropagated plantlets revealed that treatment effect (both basal media, type of PGR, concentration of PGR) was highly significant for the parameters recorded as well as their interaction ($P \leq 0.0001$) (Table 3).

The Sidak's post-hoc test ($P \leq 0.05$) was applied to explore the paired difference between the treatment means in MS media supplemented with each PGR (Kn, BAP) and basal medium (MS, WPM) while controlling the experiment-wise (family) error rate for all parameters and are depicted in Supplementary Fig. 1. It is clear from this analysis that shoot regeneration in MS was statistically comparable ($P \leq 0.05$) in 8 combinations of Kn and BAP while in the other five treatments Kn and BAP gave significant differences. Contrarily in WPM media, the analysis of shoot regeneration was statistically comparable in 12 treatments of Kn and BAP while in three treatments Kn and BAP gave significant differences.

Similarly for number of nodes developed on MS media supplemented with Kn and BAP, 10 treatments gave statistically comparable results while in other five Kn and BAP gave significantly better results (Supplementary Fig. 2). Whereas in WPM media supplemented with Kn and BAP the analysis of number of nodes was statistically comparable among all the treatments and no significant difference was observed (Supplementary Fig. 2). For number of leaves all the treatments gave statistically comparable results among MS and WPM media supplemented with Kn and BAP respectively, except one treatment (K4B4-K5B5) was significant in MS supplemented medium (Supplementary Fig. 3).

The fundamental goal of the micropropagation is to replicate the plant as quickly as possible with more number of nodal explants, since each nodal explant will develop into a plant when re-cultured. In the present work, MS medium supplemented with 0.5 mg L^{-1} BAP yielded the highest shoot length (10.42±0.78 cm) and number of nodes (6.58±0.65) (Fig. 4B).

In the present investigation, all explants cultured on BAP-supplemented media (both MS and WPM) exhibited callus formation at varying rates at the cut end (Table 1). Similar to the present study Bhat *et al.*(1995) observed a fast growing yellowish callus in *Piper nigrum* when cultured on medium containing 0.2 mg L^{-1} of BAP along with 1 mg L^{-1} of NAA. Hussain *et al*. (2011) obtained the best callus on MS media supplemented with 1.5 mg L^{-1} of BAP and the shoot regeneration in 0.5 mg L^{-1} of BAP. Kadam *et al.* (2020) observed maximum shoot induction (93.33 %) using nodal explants in MS media comprising of 4.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA.

A ratio of 1:6.5 numbers of nodes is obtained on the MS medium supplemented with 0.5 mg L^{-1} of BAP within three months. If this multiplication rate is maintained, 1,300 plants can be raised in a year from a single nodal explant. There are previous reports on *Piper in vitro* multiplication but none of the experiments tested for more than one basal media along with different concentration of PGRs as analyzed in the present study. Also, rate of shoot multiplication in the present work is better than the previously published reports on *Piper* species. Callus mediated shoot regeneration was described in *Piper colubrinum* by Kelkar *et al*. (1996), shoot buds were induced (7.6 shoots/explant) and elongated on half-strength MS supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA using leaf explants. Zhang *et al*. (2008) induced aseptic cluster shoots (6-8 shoots) on

Table 2. Effect of Murashige and Skoog (MS) medium in different strengths along with different concentration of Indole-3-butyric acid (IBA) on *in vitro* rooting of *Piper nigrum* L. shoots

Sl. No.	Composition	Rooting (%)	Number of roots/shoot	Root length (cm)
1	Full strength MS+ IBA (0.5 mg L^{-1})	100	3.25 ± 0.70^{d}	2.99 ± 0.58^{a}
2	Full strength MS+ IBA (1.0 mg L^{-1})	100	10.08 ± 1.33^{b}	3.06 ± 0.86^{a}
3	Full strength MS+ IBA (1.5 mg L^{-1})	100	6.83 ± 0.82^{c}	3.40 ± 0.30^{a}
4	**Full strength MS + IBA (2.0 mg L^{-1})**	**100**	**15.00 ± 2.04^{a}**	**3.40 ± 0.28^{a}**
5	Full strength MS	25	0.50 ± 0.26^{d}	0.96 ± 0.52^{b}
6	Half strength MS	8.33	0.08 ± 0.08^{d}	0.25 ± 0.25^{b}

Means ± SE within a column followed by the same letter are not significantly different at $p \leq 0.05$

Table 3. ANOVA of data presented in Table 1

Factors	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	1164	23	50.60	F (23, 792) = 22.31****	P<0.0001
Column Factor	2274	2	1137	F (2, 792) = 501.2****	P<0.0001
Interaction	640.4	46	13.92	F (46, 792) = 6.137****	P<0.0001
Residual	1797	792	2.268		

MS medium supplemented with 0.5 mg dm^{-3} IAA and 0.5 mg dm^{-3} BA of *Piper methysticum* using auxiliary buds as explants.

Induction of in vitro *rooting*

Rooting of black pepper shoots on the rooting media combinations is presented in Table 2. The rooting of the shoots is a prerequisite for their further establishment in the field conditions. Rhizogenesis was observed in 100% shoots on all the media combinations having IBA but their length and number varied across different concentrations. Absence of IBA in MS full- and half-strength media had lower rooting (8.33 to 25%), root length and the number of roots compared to the MS medium supplemented with IBA. Highest number of roots (15.00±2.04) and highest root length (3.40±0.28) were observed on MS supplemented with 2.0 mg L^{-1} of IBA (Fig. 4C & 4D). Earlier reports suggested development of 8 to 10 roots/ shoot when it was cultured on half-strength MS media (Philip *et al*., 1992) and 2-4 roots were observed when transferred to media containing 1µm IAA (Bhat *et al*., 1995). Rajasekaran and Mohankumar (1997) observed 3-5 roots in *Piper nigrum* on full strength white's media without any growth regulators after 14 days of inoculation. Ahmad *et al.* (2014) observed higher rooting percentage (90%) on IBA (1.5 mg L^{-1}) in black pepper. Salim *et al.* (2017) obtained 80 % rooting of *P. nigrum* when cultured on half-strength MS medium containing 1.5 mg L^{-1} IBA. Ramos *et al.* (2020) observed number of roots (6.40) in "Clonada" genotype in after 8 wks of inoculation when cultured on MS medium with 0.05 mg L^{-1} NAA in black pepper.

The present study provides an evident protocol with 100% rooting, highest number roots/shoot (15.00±2.04) and root length (3.40±0.28) when compared to the earlier studies establishing the superiority of present protocol to develop robust plantlets of black pepper.

Acclimatization to ex vitro *conditions*

The ultimate success of *in vitro* propagation lies in the successful establishment of plantlets in the field conditions. In the present study plantlets showed 100 % survival in mist chamber at the primary hardening stage. The plantlets resumed the growth within 3 wks (Fig. 4E). After 4 wks the hardened plants were planted in earthen pots and were placed under shade net condition (Fig. 4F). The different stages of micropropagation protocol standardized in the present work is depicted in Fig. 5.

Genetic fidelity studies using ISSR marker

Many researchers have demonstrated the value of molecular analysis of *in vitro* regenerated plants (Bhatia *et al*., 2011; Saha *et al*., 2016). The genetic stability of micropropagated plants has enormous practical utility and commercial value since it provides information about the structural and functional stability of the plants that have been regenerated. With this context in mind, the molecular analysis was undertaken using the ISSR markers, by comparing the mother plants with the regenerated plants.

Table 4. Primer sequence and number of scorable bands produced by ISSR primers in *Piper nigrum* L. in mother plant and micropropagated plantlets

Sl. No.	Primer name	Tm(°C)	Primer sequence (5^1-3^1)	Total number of scorable bands
1	UBC 801	37	$(AT)_8T$	0
2	UBC 802	37	$(AT)_8G$	0
3	UBC831	37	$(AT)_8TA$	0
4	UBC 832	37	$(AT)_8TC$	0
5	UBC 833	37	$(AT)_8TG$	0
6	IS10	49.2	$C(GA)_8$	4
7	UBC 860	49.2	$(TG)_8GA$	0
8	UBC 814	50	$(CT)_8A$	1
9	UBC 819	50	$(GT)_8A$	0
10	UBC 820	50	$(GT)_8C$	0
11	UBC 836	50	$(AG)_8TA$	3
12	UBC 843	50	$(CT)_8GA$	0
13	UBC 829	50	$(TG)_8C$	3
14	UBC 840	50	$(GA)_8TT$	0
15	UBC 824	50	$(TC)_8G$	0
16	UBC 834	50.6	$(AG)_8TT$	3
17	UBC 835	50.6	$(AG)_8TC$	2
18	IS7	51	$(GT)_8A$	0
19	UBC 858	51	$(TG)_8GT$	5
20	IS8	51	$(AG)_8C$	4
21	UBC 870	51	$(TGC)_6$	4
22	UBC 871	51	$(TAT)_5$	0
23	UBC 872	51	$(GATA)_3GAT$	0
24	UBC 859	51	$(TG)_8GC$	0
25	UBC 842	51	$(GA)_8TG$	4
26	UBC 813	52	$(CT)_8T$	0
27	IS11	52	$(CA)_7G$	5
28	IS65	52	$(AG)_8T$	3
29	UBC 826	52.7	$(AC)_8C$	4
30	UBC 825	52.7	$(AC)_8T$	2
31	IS 12	52.7	$(GT)_8C$	0
32	IS 9	53.5	$(TG)_8A$	2
33	UBC 873	53.5	$(GACA)_4$	0
34	UBC 841	53.5	$(GAGA)_4TC$	4
35	IS 53	54	$(GA)_8C$	2
36	IS 61	54	$(GA)_8T$	3
37	UBC 861	56.6	$(ACC)_6$	4
38	UBC 856	50	$(AC)_8CA$	3
39	UBC 846	50	$(CA)_8AT$	3
40	UBC 847	50	$(CA)_8AC$	3
41	UBC 848	50	$(CA)_8AG$	5
42	UBC 849	50	$(GT)_8CA$	2
43	UBC 850	50	$(GT)_8CC$	2
44	UBC 851	50	$(GT)_8CG$	3
45	UBC 852	50	$(TC)_8GA$	0
46	UBC 853	50	$(TC)_8AT$	0
47	UBC 854	50	$(TC)_8$ AG	0
48	UBC 855	50	$(AC)_8$ CT	3
			TOTAL	86

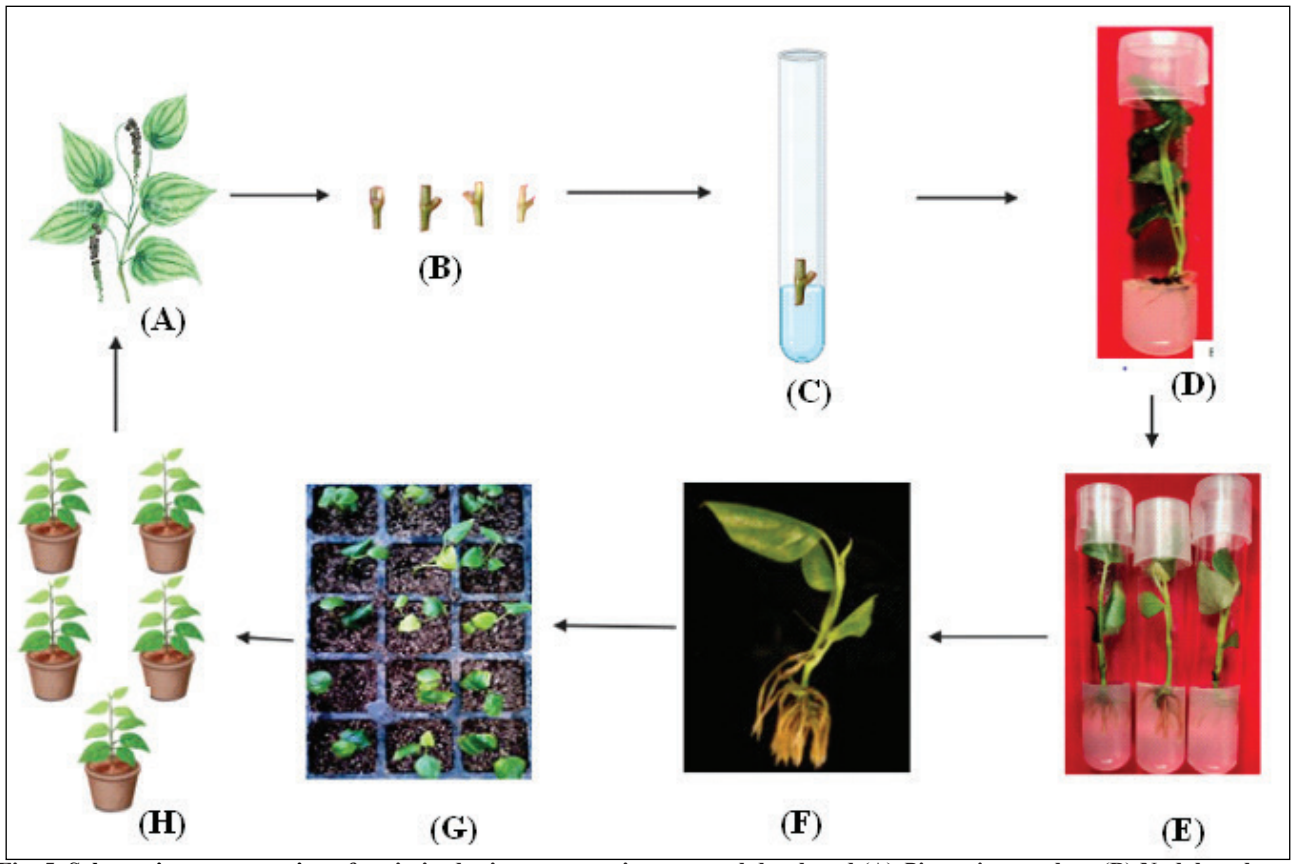

Fig. 5. Schematic representation of optimized micropropagation protocol developed (A) *Piper nigrum* plant (B) Nodal explants used (C) Culturing of the nodal explants on different media combinations (D) Cultured plants on MS media supplemented with 0.5 mg L^{-1} BAP (E & F) Plants rooted on MS media supplemented with 2.0 mg $L^{-1}$of IBA (G) Hardening of plants in mist chamber (H) Transferring of hardened plants to pots containing 1:1 ratio of soil and farm yard manure (FYM)

Genetic stability was assessed by comparing DNA isolated from mother stock cultures, plantlets regenerated on K2 medium (MS + 0.5 mg L^{-1} Kn) and B2 medium (0.5 mg L^{-1} BAP). Higher callus was recorded at the cut ends of the shoot in B2 medium as compared to K2, hence the comparison for genetic stability between the two media was considered prudent. Only 29 out of 49 markers examined yielded clear and reproducible bands. The optimal annealing temperature was found to be between 37 °C to 56.6 °C (Table 4). The 29 ISSR markers resulted in 86 distinct and scorable bands with sizes ranging from 120 (UBC842) to 1,200 (UBC841). The number of scorable loci ranged from 1 (UBC 814) to 5 (UBC 858 and IS11), with an average of 3.18 loci per primer. The maximum number of loci was restricted to the ladder range of 300 to 1000 base pair. Among the 29 markers examined, all the generated loci were found to be monomorphic. Furthermore, no difference in banding pattern was observed between the micropropagated and mother plants (Fig. 6). This genetic stability, in turn, indicates the suitability of this protocol for large-scale commercial propagation within three months. Similar results have been reported by Malhotra *et al*. (2020) in cardamom plants.

Conclusion

A two-step improved and a reproducible micropropagation protocol of black pepper is presented in this study. The medium comprising full-strength MS basal media supplemented with 0.50 mg L^{-1} of BAP was the most appropriate to produce highest number of nodes and shoot length in unit time. The developed shoots were vigorous and rooted well on MS media supplemented with 2.0 mg L^{-1} of IBA. This protocol regenerated plantlets which are genetically stable as assessed by ISSR markers. Because of its high economic value and its trade across the countries, a tissue culture protocol with good rate of multiplication and maintenance of genetic stability will be useful for commercial application of the developed protocol.

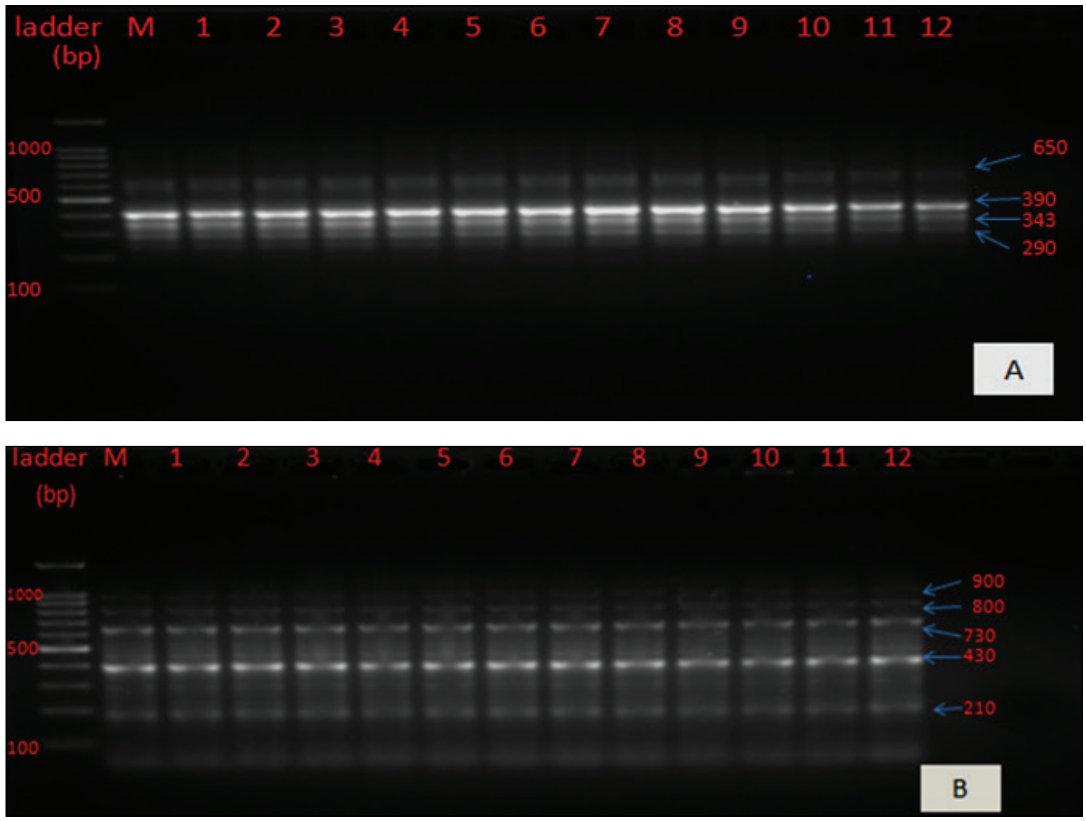

Fig. 6. ISSR banding profiles of obtained with ISSPR primer IS 8(A) and UBC 848 (B) of the mother plant and micropropagated plants from B2 medium (Ladder lane: 100 bp DNA marker, M- mother plant, 1-12: micropropagated plants)

Acknowledgements

DDA thanks ICAR-IARI for PhD Fellowship. We thank Director, ICAR-NBPGR, New Delhi, for providing the necessary laboratory facilities.

Author Contributions

DDA carried out all the experiments and wrote the draft manuscript. AA provided overall supervision for the work and critically edited the manuscript. EVM designed the experiments and provided guidance. DDA and MS carried out statistical analysis. All authors have read and approved the manuscript.

*Supplementary Table or Figure mentioned in the article are available in the online version.

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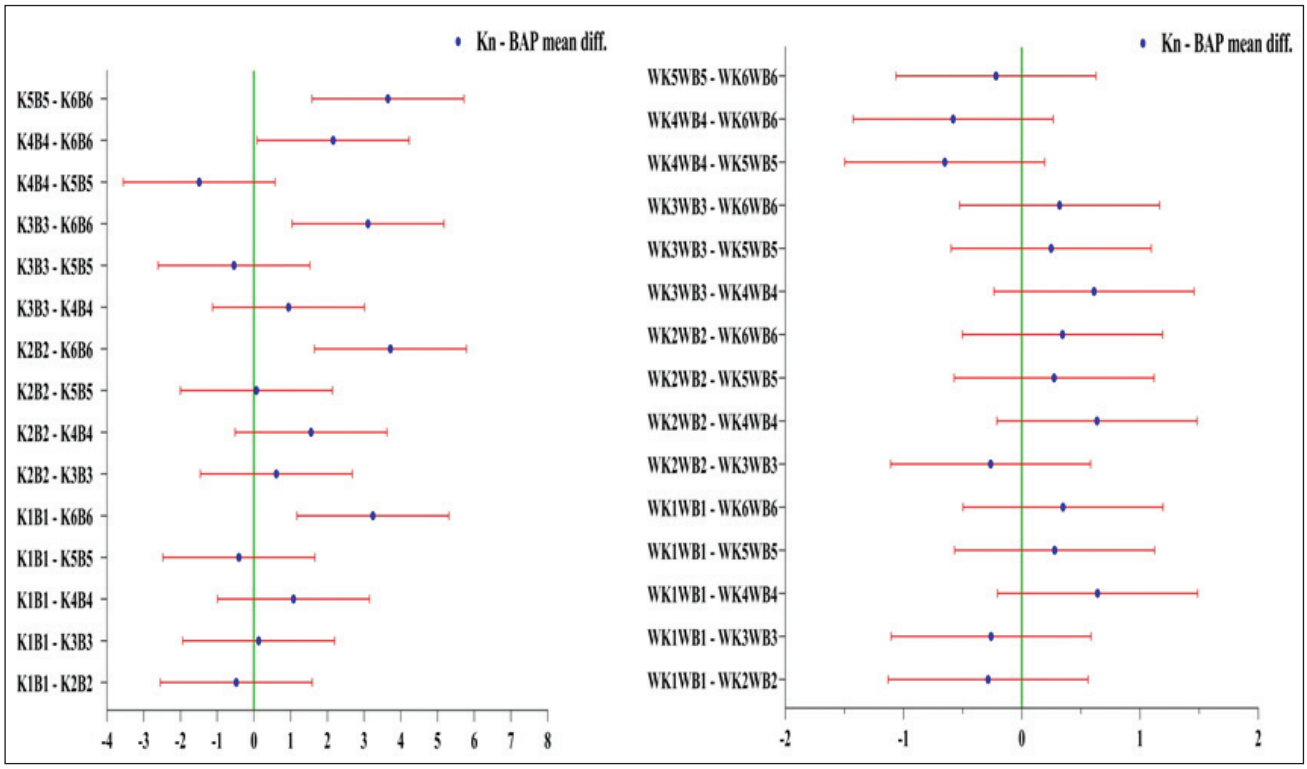
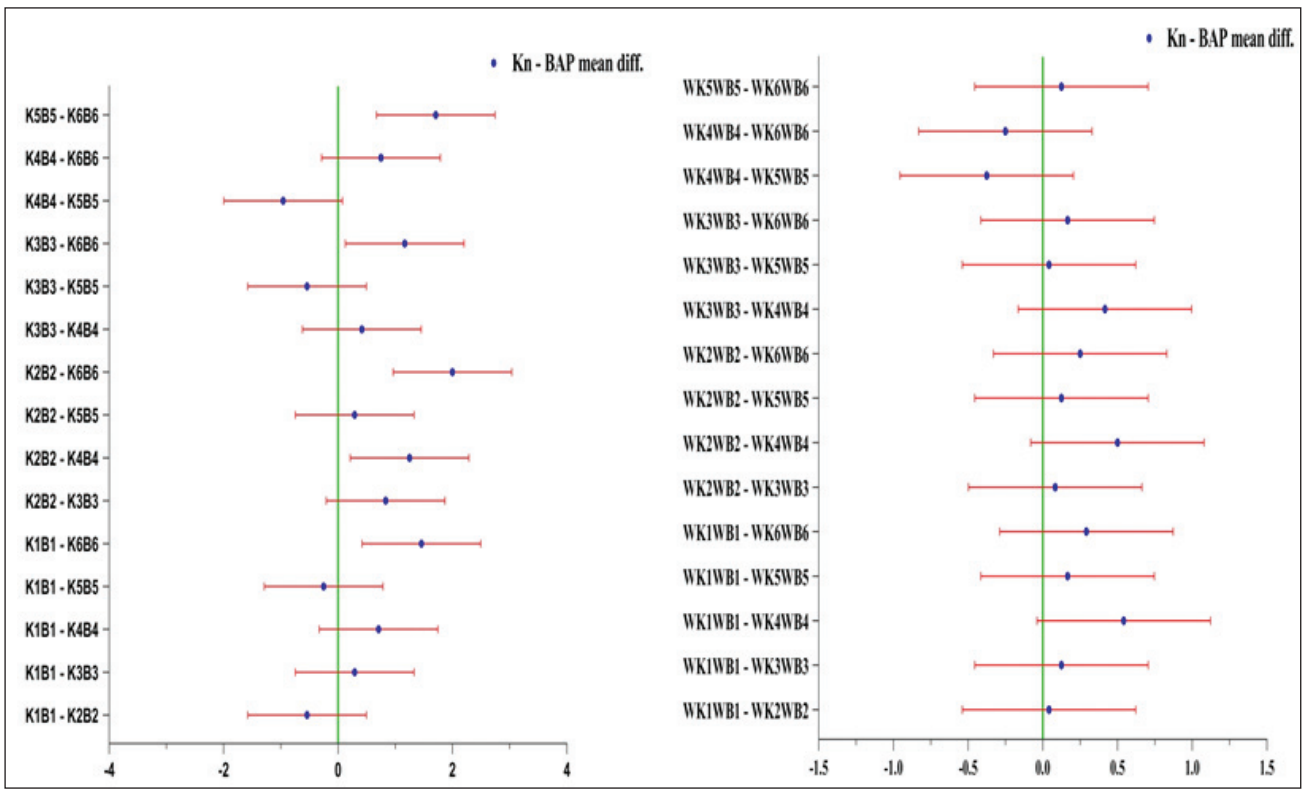
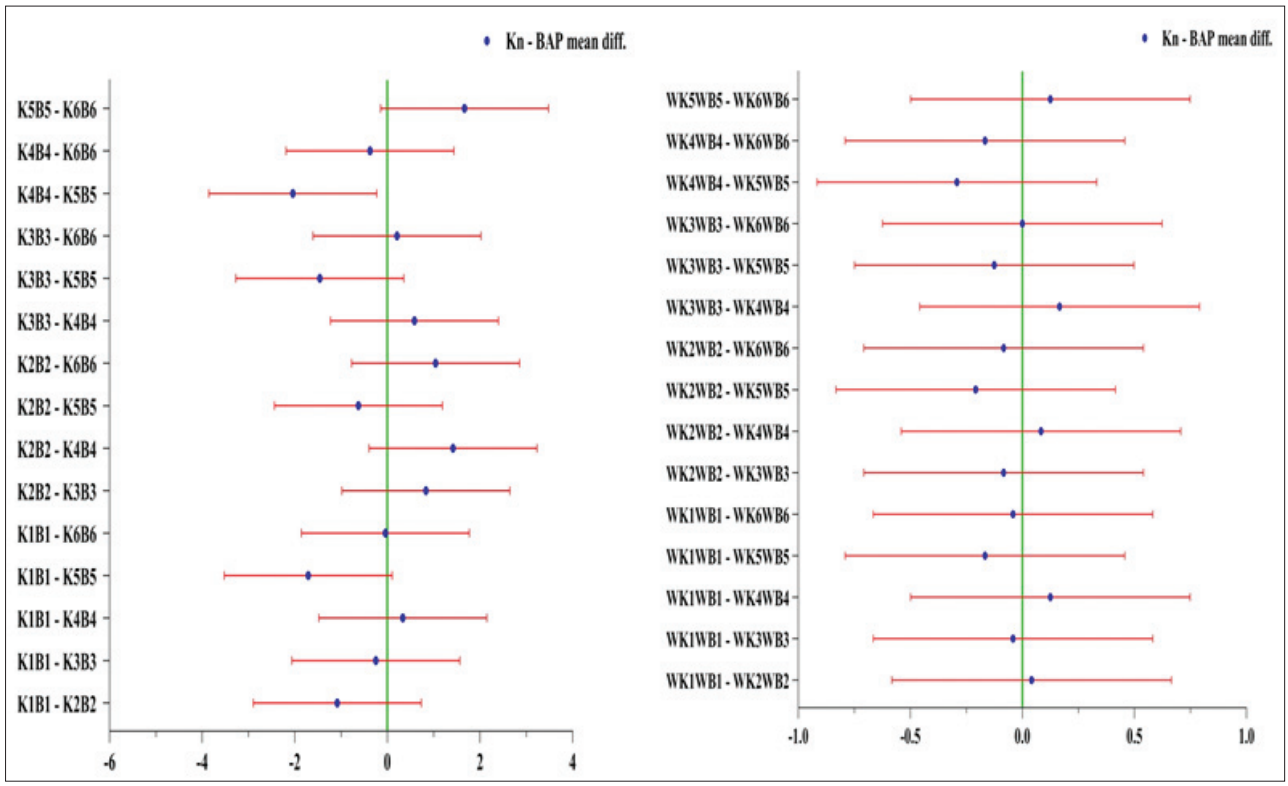


Supplementary Fig. 1. Paired comparison of *Piper nigrum* L. shoot length for Kn and BAP in MS and WPM media using post-hoc Sidak's multiple comparison test (for media codes refer to Table 1).



Supplementary Fig. 2. Paired comparison of *Piper nigrum* L. number of nodes for Kn and BAP in MS and WPM media using post-hoc Sidak's multiple comparison test (for media codes refer to Table 1).



Supplementary Fig. 3. Paired comparison of *Piper nigrum* L. number of leaves for Kn and BAP in MS and WPM media using post-hoc Sidak's multiple comparison test (for media codes refer to Table 1).

GUIDELINES TO AUTHORS

GENERAL

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ORGANIZATION OF THE MANUSCRIPT

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Pusa Campus, New Delhi–110012, India

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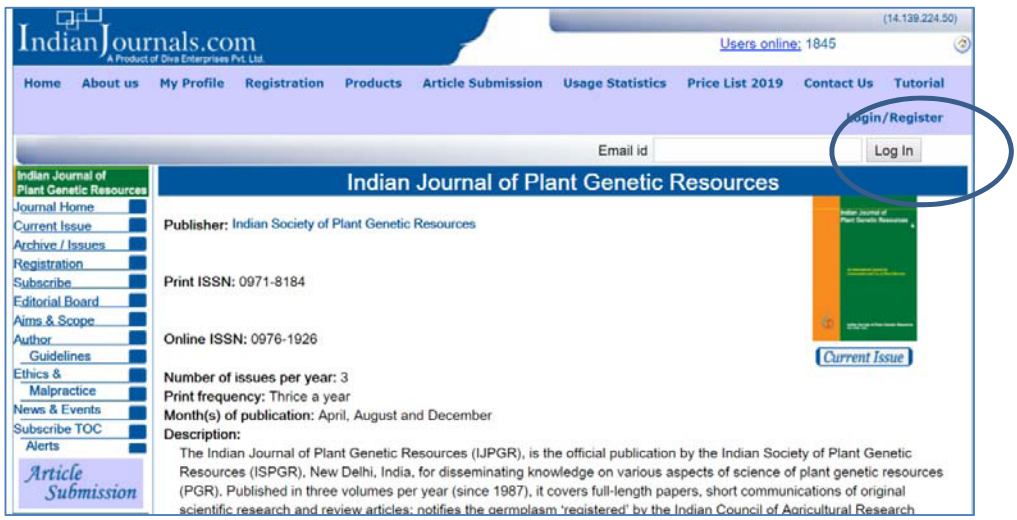

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Print ISSN: 0971-8184
Online ISSN: 0976-1926

Indian Journal of Plant Genetic Resources

Vol. 35 No.2 2022

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Published by the *General Secretary*, Indian Society of Plant Genetic Resources, NBPGR Campus, New Delhi-110012 and printed at *Angkor Publishers (P) Ltd*., B-66, Sector 6, NOIDA. Mobile: 9910161199, E-mail: angkor@rediffmail.com.