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RESEARCH ARTICLE

ISOLATION AND SEQUENCE ANALYSIS OF NBS-LRR ENCODING GENE ANALOGS IN TWO SPECIES OF *DRIMIA*

H. M. HANUMANTHA RAO AND L. RAJANNA*
Department of Botany, Bangalore University, Jnanabharathi Campus, Bengaluru 560 056
*For correspondence. Email: lrajannabot@gmail.com
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SUMMARY Nucleotide binding site-leucine rich repeats (NBS-LRR) encoding nucleotide sequences in *Drimia indica* and *D. polyphylla* have been studied by using degenerate polymerase chain reaction (PCR) method. The sequences were compared with known NBS-LRR type R-genes of crop plants. *D. indica* genomic DNA produced NBS specific PCR amplified product. The phylogenetic tree analysis showed that isolated *D. indica* NBS encoding nucleotide sequences are grouped separately and slightly distant from that of the other crop sequences. The protein motif analysis revealed that *D. indica* NBS encoding sequences belong to subclass CC-NBS-LRR type. Possible pseudogene family occurrence in *D. polyphylla* genome restricts the amplification of specific NBS domain encoding nucleotide sequences.

Keywords: *Drimia*, NBS-LRR type R-genes, degenerate PCR, nucleotide sequences, protein motif.

INTRODUCTION

Drimia indica and *D. polyphylla* are perennial herbs having a bulbous geophyte belong to the family Asparagaceae. To resolve their morphological similarities and genomic complexities of Indian *Drimia* species, Desai et al. (2012) used DNA sequence of 2 molecular markers such as "internal transcribed spacers (ITS)" and "maturase K (matK)" disclosed that *indica* complex and *wightii* complex are evolving parallelly by grouping them under 2 clusters which confirms that the conventional and molecular methods are significant in complex group of *Drimia* species. Similarly, Jehan et al. (2014) used RAPD and SRAP markers to explore genetic diversity of 3 genera namely, *Drimia*, *Dipcadi* and *Ledebouria* of Hyacinthaceae. This study was successful in obtaining species-specific markers and showed good correlation in overall data. Lekhak et al. (2017) made a cytogenetic study and confirmed the presence of many cytotypes at the population level. Further, to overcome the limitations of morphological markers in diversity analysis Yadhav et al. (2019) collected the data from palynology, meiotic observations and inter-specific hybridizations to recognize the 9 Indian species in the genus.

Disease resistance is an important mechanism in all living organisms. The NBS-LRR type R-genes will be considered as functional conserved DNA markers (CDMs) to explore

disease resistance genes in the plant systems (Rob Van Treuren 2005, Spooner 2005). Because, most of the disease resistance genes in plants encode nucleotide-binding site-leucine rich repeat (NBS-LRR) proteins (McHale et al. 2006). Unlike RAPD markers, functional marker characterizations deliver both diversity and functionality of the specific plant simultaneously. The studies involved in isolation and characterization of NBS-LRR genes have been limited to only economically important crop plants. The role of NBS-LRR gene in disease resistance mechanism is complex and not completely known in many plants (McHale et al. 2006). Therefore, to understand the structure and mechanism of R-gene, defence mechanisms in different plants and NBS-LRR genes must be studied. Hence, the present study aims to explore the NBS-LRR resistance gene analogs in *D. indica* (Roxb.) Jessop and *D. polyphylla* (Hook.f.).

MATERIALS AND METHODS

The plant materials were collected from Castle rock and Tataguni estate, Aagara village, Bengaluru rural and maintained in the greenhouse of the Department of Botany, Bangalore University. Leaf samples of *D. indica* and *D. polyphylla* were collected and immediately stored at–80°/ C. The genomic DNA of leaf samples were extracted by using column-based thermo-scientific GeneJET plant genomic DNA purification mini kit. The extracted DNA was quantified using nanodrop spectrophotometer and the integrity was checked by resolving 7 l eluted DNA sample in 1% agarose gel and subjected to electrophoresis at 100 V for 30 min. Isolation of NBS-LRR nucleotide sequences was carried out by using degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) and TA cloning method. The universal degenerate primer set was designed for conserved P-loop (LM638) motif and GLPL motif (LM637) within the NBS domain using qualified NBS-LRR type R-genes from different plants (LM638 5′– GGIG GIGTIGGIAAIACIAC–3′ and LM637 5′– ARIGCTARIGGIARICC–3′) (Kanazin et al. 1996, Miller et al. 2008). The PCR was carried out using GeNei laboratories taq DNA polymerase kit (Cat. No: 0601600051730). 2 l of 10 x taq buffer, 3 l of 2.5 mm dNTP mix, 1.5 l of 10 picomol forward primer, 1.5 l of 10 picomol reverse primer, 0.4 l of taq polymerase, 2 l of (50 to 100 ng) of genomic DNA and 9.6 l of PCR grade nuclease-free water were added to make up final reaction volume of 20 l. The PCR programme was set at 95° C for 10 min initial denaturation, 95° C for 1 min secondary denaturation, 48° C for 1 min annealing and 72° C for 1.5 min extension with 35 cycles and 72° C for 15 min final extension. *D. polyphylla* NBS-LRR nucleotide sequences were amplified by slightly modified amplification protocol. The final reaction volume of 20 l contains all required component as mentioned above subjected to gradient PCR method; the programme set at 95° C for 10 min initial denaturation, 95° C for 1 min secondary denaturation, the gradient annealing temperature option was set to obtain temperature ranging from 37° C to 54° C for 1 min and 72° C for 1.5 min extension with 35 cycles and 72° C for 15 min final extension.

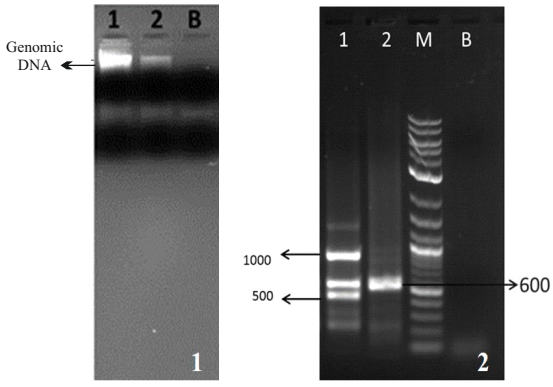
The 500 bp and 600 bp PCR bands were excised from the agarose gel using gel extraction kit (Cat. No: K0691). Each PCR fragment was cloned into the plasmid using the pGEM-T easy vector system (Cat. No: A1360) and transformed

into competent *Escherichia coli* strain, JM109. The transformed cells were screened using x-gel containing selection media plates. The transformed patch plated on Luria-Bertani (LB) agar plates. The plasmid of an insert containing colonies were isolated using a gene jet plasmid isolation kit (Cat. No: K0502).

DNA sequencing was conducted at Eurofins Scientific India Pvt Ltd, Bengaluru, India. The primary amplification was conducted using vector specific T7 RNA polymerase promoter Primer, sequencing cycle was performed using big dye terminator v3.1 kit (di-deoxy chain termination method), and capillary electrophoresis was conducted by using 3730 xL genetic analyzer applied biosystems, USA. The DNA sequence quality was checked by using chromos software and vector contamination was removed by using NCBI vecscreen tool. The NCBI-BLASTx tool integrated to NCBI-ORF finder was used for sequence homology search. Polymorphism in the selected sequence was analyzed by DnaSP version 6 software. The NCBI-ORF finder software and translate tool-ExPASy was used to convert DNA to possible amino acid sequence. Amino acid motif analysis was carried out by using CLUSTALW multiple sequence alignment programme and edited with the JALVIEW programme.The MEGAx software was used to generate phylogenetic tree and diversity investigation. The conserved motif analysis associated phylogenetic tree was inferred using the neighbor-joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 5.18381572 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the poisson correction method (Zuckerkandl & Pauling 1965) and are in the units of the number of amino acid substitutions per site. This analysis involved 9 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1874 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

OBSERVATIONS

The column-based DNA isolation method yielded a good amount of DNA in *D. indica* but the DNA concentration was very low in *D. polyphylla*. The nano drop spectrophotometer was used to quantify the extracted DNA which showed 339 ng/l for *D. indica*, 176 ng/l for DNA for *D. polyphylla.* The DNA integrity check was done by using 1% agarose gel (Fig. 1). The degenerate PCR of *D. indica* and *D. polyphylla* for amplification of NBS encoding nucleotide sequences were performed with different PCR programme conditions. *D. indica* yielded 3 prominent bands at 500, 600 and 1000 bp in agarose gel electrophoresis (Fig. 2). In contrast, *D. polyphylla* failed to produce the amplification with the same PCR condition. Further, to amplify NBS specific bands in *D. polyphylla* the PCR was standardized by using gradient PCR method with different range annealing temperature from 37° C to 54° C for 1 min. After the gradient PCR conditions *D. polyphylla* showed a single prominent band at 600 bp from annealing temperature 37° C to 46° C (Fig. 2). The results of TA cloning of PCR product obtained from



Figs 1 & 2: 1. DNA integrity check in 1% agarose gel. Lane 1. Genomic DNA of *D. indica*. Lane 2. Genomic DNA of *D. polyphylla.* 2. Agarose gel electrophoresis showing PCR band of *D. indica* and *D. polyphylla*, M. 0.1 to 10 kbp marker, 1. *D. indica* (Tataguni) 600/500 bp. 2. *D. polyphylla* (Pune) 600 bp.

D. indica and *D. polyphylla* are given in Table 1. 17 out of 26 colonies were selected from *D. indica* and 8 out of 13 colonies were selected from *D. polyphylla* for plasmid isolation and sequencing. Finally, 6 diverse sequences from *D. indica* (3 500 bp and 3 600 bp) and 3 diverse 600 bp sequences from *D. polyphylla* were selected for further analysis. The nucleotide sequence of *D. indica* and *D. polyphylla* were searched for potential protein encoding segments by using NCBI-open reading frame finder tool and BLAST programme (Table 2). The ORF finder parameters were set to use "ATG" and "alternative initiation codons" to disclose all ORFs in the query sequences. Both 500 and 600 bp sequences were used for analysis. The disclosed ORFs amino acid sequences were used to check the homology match between predicted proteins by using the smart basic local alignment search tool (BLAST) and regular BLASTp tools. The *D. indica* 500 bp sequences yield a similar BLAST result for all 3 nucleotide sequences. The 500 bp sequences yield 3 ORFs and smart BLAST results were matched with predicted CC-NBS-LRR class R-genes of *Arabidopsis thaliana.* But 600 bp sequences disclosed one ORF in positive strand with reading frame 2, however, the BLAST results did not match with any disease resistance proteins. Also, the expasy-translate results of 600 bp of *Drimia* species revealed the presence of irregular stop codons in the sequences; hence, 600 bp sequences are not taken for further analysis. The DNAsp software polymorphism analysis of 3 500 bp

TABLE 1**:** Cloning results of *D. indica* and *D. polyphylla*.

Cloning and sequencing steps followed	*D. indica* (Both 600 bp and 500 bp PCR product)	*D. polyphylla* (600 bp PCR product)
Blue–white screening for transformed cells	26 well isolated white colonies obtained	13 well isolated colonies obtained
Patch plate on 5 × 5 square grid	17 colonies	8 colonies
Subculture and plasmid isolation	17 plasmids extracted	8 plasmids extracted
Sanger sequencing of plasmids	All 17 plasmids sequenced with T7 promoter primer	All 8 plasmids sequenced with T7 promoter primers
Sequence quality check and removing identical nucleotide sequences form group	Total 6 sequences selected for analysis (3 diverse 500 bp sequence and 3 600 bp sequence)	1

TABLE 2: Results of NCBI–ORF finder and protein prediction using SMART BLASTp for 500 and 600 bp sequences of *D. indica*. The last row contains Expasy–Translate Tool results showing irregular stop codon.

Drimia sequences	ORFs	Frame	Strand	Start	Stop	Amino acid sequence	Smart Blast results
500bp amplified PCR band	ORF 1	1	Positive	97	303	>lcl|ORF1 MASRDCTVEKLQDQV VKRLDLKDREQIFSYL SNKSFVLLLDDVWDP LDLKRVDSLFLLAPSA KANDVS	Disease resistance protein (CC–NBS–LRR class) family [*Arabidopsis thaliana*] [NP_175559.2] P–loop containing nucleoside triphosphate hydrolases superfamily protein [*Arabidopsis thaliana*] [NP_188191.1] RPS5–like 1 [*Arabidopsis thaliana*] [NP_172685.1] NB–ARC domain–containing disease resistance protein [*Arabidopsis thaliana*] [NP_194339.1]
500bp amplified PCR band	ORF 2	2	Positive	68	145	>lcl|ORF2 MLGSIVSSLLWLPEIA RLKNFKTKW	Putative leucine–rich repeat receptor–like serine/threonine–protein kinase At2g19230 [*Zingiber officinale*] [XP_042465196.1] XP_010239691.1|disease resistance protein RPM1 [*Brachypodium distachyon*] XP_022002538.1|putative disease resistance RPP13–like protein 1 [*Helianthus annuus*] Disease resistance protein (CC–NBS–LRR class) family [*Arabidopsis thaliana*] [NP_001318986].
500bp amplified PCR band	ORF 3	3	Positive	300	>500	>lcl|ORF3 MILTTRSQHVCDNIEV HNISEVKCLSHEAARR LFRKKVSEDAINSHPM LPKLIDKIADECNGLP LAL	Select seq ref|XP_003533606.3| probable disease resistance protein At5g63020 [*Glycine max*] [XP_003533606.3] LRR and NB–ARC domains–containing disease resistance protein [*Arabidopsis thaliana*] [NP_192816.1]
500bp amplified PCR band	ORF 1	2	Positive	77	340	>lcl|ORF1 MGLDAKELEACPSPII DFTRHSVLVLGMISLP IALESHPRVATRMIKF LVVGVNSAYNRIIDRP LLNSIKVVVTPLHHMI MFRIEG	|XP_009383139.1|PREDICTED: uncharacterized protein LOC103970955 [*Musa acuminata subsp. malaccensis*]
600bp amplified PCR band	Results output of "Translate tool – Expasy" for the 600bp sequences			# 5'3' Frame 1 G G V G K T T N G Stop Stop R Y S Met Stop C I I H I S I L K D G S R R K G V R S L P Stop P Y Y Stop F H Stop T Stop R P G A W D D L T T Y R T R E S P E G G H E N D Stop V F G R R G Stop L C L Q Q D H R S T I A Stop L Y Q G R C H T T S S H D Y V S Y R G G I R K V K G S Q S Q S R L C Y Q A S N K L Stop S P S P N L P S D E K K Q K K G K S V L I E E K I T Y L V A Met L D V Stop G E L Q E K R T E P T E D I K T V T Met P K L N H N L I H L G G F P L A (Stop– presence of stop codons)			

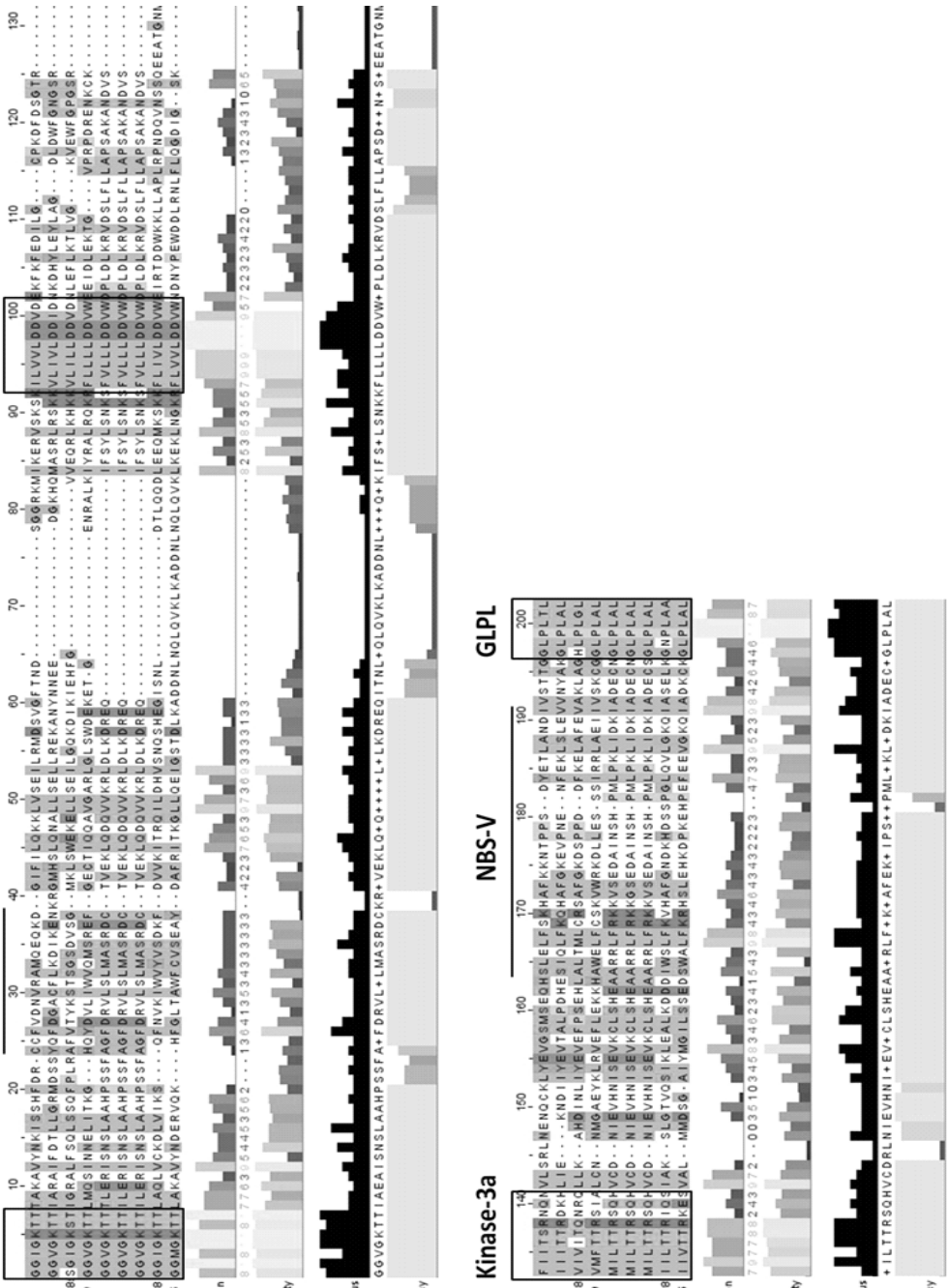


Fig. 3: Alignment of deduced amino acid sequences of 3 RGAs of *D. indica* and 6 qualified NBS-LRR type R-genes (Tobacco N, Tomato I2 and M, *Arabidopsis* RPP5, Rice Xa1 and RPS2 *Arabidopsis)*. Identical amino acids sequences used for CLUSTAL2 and JALVIEW representation. Similar amino acid sequences are blocked with the same colour and conserved motifs are shaded and labeled as described in the Pan et.al 2000 and Palomino et.al 2006.

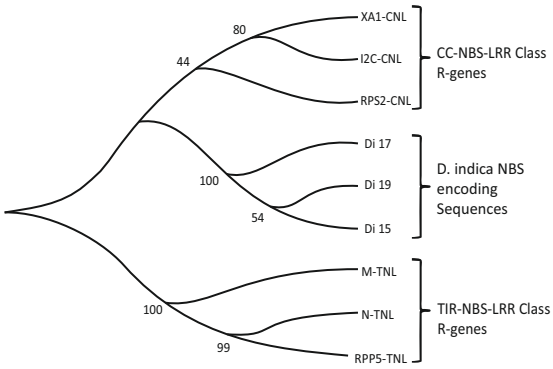


Fig. 4: Phylogenetic relationship of amino acid sequences of 3 RGAs isolated form *D. indica* and six characterized NBS-LRR Type R-genes from *Arabidopsis* (RPS2, RPS5), Rice (Xa1), Tomato (I2C, M) and Tobacco (N) plants.

sequences of *D. indica* showed 5 polymorphic sites with 5 mutations in the nucleotide sequences. The gene diversity Hd was 1 and variance of gene diversity was 0.07407. These nucleotide sequences were submitted to GenBank and obtained GenBank accession numbers, ON921720, ON921721 and ON921722. Conserved motif analysis of *D. indica* was conducted by comparing amino acid sequences with qualified NBS-LRR class R-genes sequences from different plant species. The clustlw tool was used to generate multiple sequence alignment and jalview programme was used to edit and highlight the conserved motifs (Fig. 3). The results showed that *D. indica* NBS encoding sequences maintain a good conservation at p-loop motif (GGVGKTT), kinase-2 motif (VLDDVW) and GLPL motif in the amino acid sequence. However, the *Drimia* sequences appear slightly different from other motifs such as kinase-3a motif and NBS domains. In addition, in *D. indica*, the kinase-2 motif amino acid valine and isoleucine are replaced by leucine (V/L or I/L) at 3rd amino acid position. Leucine is replaced by valine at 2nd amino acid position in kinase-2 motif (FVLLLDDVW) (Fig. 3). The kinase-2 motif of *Drimia* was used for homologue search against predicted proteins database. The results showed that *D. indica* kinase-2 motif was matched 100% to disease resistance protein RFL1-like isoform X1 (*Vitis riparia*) (XP_034680452.1). In the sequence information, the RFL1-like isoform X1 is noted as resistant to *Pseudomonas syringae*. Also, when compared to qualified R-gene used in this study, the *Drimia* kinase motif matches to 90% with RPS2 R-gene. Interestingly, the disease resistance protein RFL1 alternative name is RPS5-like protein 1. In phylogenic tree analysis along with the *D. indica* protein sequence, 3 TIR-NBS-LRR(TNLs) and 3 CC-NBS-LRR(CNLs) subtypes qualified R-genes were included for comparison (Fig. 4). As expected, CNLs and TNLs were grouped separately in the phylogenetic tree. *D. indica* - RGAs shared the branch with CNL, but grouped distantly as separate population. In addition, protein motif analysis confirmed that the last amino acid combination of *D. indica*-specific kinase motif-2 consists of amino acid "Tryptophan" which confirms that isolated NBS encoding nucleotide sequence is CNL subtype.

DISCUSSION

The present findings are similar to those obtained in *D. wightii (*Hanumantha Rao et al. 2022). While DNA obtained from *D. polyphylla* is lesser as compared to *D. indica* due to the presence of high mucilage content in leaf sample. Degenerate primers amplified NBS-specific PCR fragment in *D. indica* but failed to amplify specific NBS fragments in *D. polyphylla,* instead PCR

produced nonspecific 600 bp fragment. The BLAST results of *D. indica* 500 bp sequences were successfully matched with the NBS-LRR type R-genes, but 600 bp fragment sequences did not match with any disease resistance genes in both *D. indica* and *D. polyphylla*. Further analysis of 600 bp sequences confirmed the presence of irregular stop codons which shows that these sequences comprise possible pseudogene-like features. Sometimes, the PCR amplification of desired gene will be affected or failed if the genome contains large pseudogene families called PCR bias or amplification bias; this might be the possible reason for PCR amplification failure in *D. polyphylla* (Chen et al. 2011). Protein motif analysis has revealed disclosed the presence of amino acid "Tryptophan" as a last residue of kinase-2 motif in the isolated *D. indica* nucleotide sequences, which confirms the occurrence of CNL subclass disease resistance family protein in the plant. *D. indica* R-gene analogs were matched with RPS5-like disease resistant protein of *Arabidopsis thaliana*. The UniProtKB - O64973 (RPS5_ARATH) recorded that RPS5 disease resistance (R) protein that specifically recognizes the avrPphB type III effector aviru-lence protein from *P. syringae* and also confers resistance against *Hyaloperonospora parasitica*. Further, the small nested ORF match with short sequences of putative disease resistance RPP13-like protein 1 (*Helianthus annuus*). The RPP13 is a simple locus in *Arabidopsis thaliana* for alleles that specific downy mildew resistance to different avirulence determinants in *Peronospora parasitica* (Bittner Eddy et al. 2000). The CNL subtype having evolutionary significance in disease resistance mechanism as compared to TNLs, because monocot genomes contain only CNL subtypes but both CNLs and TNLs are present in dicot genomes. The monocots evolved with only CNL type R-genes to defend against different biotic challenges. The polymorphism results showed that the isolated *D. indica* sequence is highly identical with only point mutations. This indicated disease resistance genes were evolving slowly with point mutations and not by rapidly evolving diverse sequences. In some cases, a small number of R-genes can provide defense against diverse pathogens if a limited number of effector targets are present (Meyers et al. 2003). Further, the pseudogenes have an importance in evolutionary biology. In comparative analysis pseudogenes provide a record of ancient genes and help to determine the rate of duplication. Furthermore, some pseudogenes play a vital role in gene regulation (Witek & Mohiuddin 2019, Yusuf Tutar 2012). The present study is limited to only PCR amplified nucleotide sequences. To know the importance of obtained *Drimia* R-gene analogs further functional studies must be conducted. The results obtained in the present study revealed that the universal degenerate primers designed for highly conserved regions of the NBS domain are efficient to amplify the NBS encoding genes in *D. indica* and also give an indication that RGAs in *D. indica* have evolved to defend the broad range pathogens.

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RESEARCH ARTICLE

A COMPARATIVE STUDY ON STOMATAL CHARACTERISTICS AND PHOTOSYNTHETIC PIGMENTS IN WILD AND CULTIVAR OF *ZAMIOCULCAS ZAMIIFOLIA* (LODD.) ENGLER

CINTHYA CHRISTOPHER*, P. S. ASWATHY AND H. HARITHA
Department of Botany, All Saints' College, University of Kerala, Chackai, Thiruvananthapuram 695 007
*For correspondence. Email: cinthya_wilfred@yahoo.com
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SUMMARY *Zamioculcas zamiifolia* (Araceae), an unusually drought resistant medicinal plant is native to tropical East Africa, and subtropical south-east Africa. It is described as a living fossil which may have evolved as early as 42 million years ago. In the present study, an attempt has been made to study the foliar morphology, stomatal variations, photosynthetic pigment estimation and absorption spectra analysis in wild and cultivar of this species. A strong negative correlation exists between the stomatal frequency and the area occupied by the stomata and between frequency of epidermal cells and the area occupied by the epidermal cells. Estimation of photosynthetic pigments revealed that the amount of chlorophyll *a* and *b*, total chlorophyll and carotenoid contents were high in the wild as compared to the cultivar. Stomatal study, estimation of photosynthetic pigments and absorption spectra analysis revealed that the wild is more efficient in its metabolic vigour as compared to the cultivar.

Keywords: *Zamioculcas zamiifolia,* stomata, photosynthetic pigments.

INTRODUCTION

Zamioculcas zamiifolia, commonly known as Zanzibar gem, Zuzu plant or Emerald palm, is a tropical perennial medicinal plant belonging to the family Araceae. It is a sole species of the genus *Zamioculcas* and native to eastern Africa, from Kenya South to northeastern South Africa. The plant is grown in dry grassland and often in stony ground and has several fleshy stalks bearing alternate pinnate leaflets and a very thick rhizome. The leaflets have the capacity to sprout new plants and form tiny rhizomes at their bases (Brown 2000). It is grown as an ornamental plant, mainly for its attractive glossy foliage. The plant contains an unusually high-water content of leaves (91%) and petioles (95%) (Moullec et al. 2015) and has an individual leaf longevity of at least 6 months (Chen & Henny 2003) which may be the reason for its survival under interior low light levels for 4 months without water.

Z. zamiifolia shows 2 defining attributes of crassulacean acid metabolism (CAM). It is the only CAM species described within Araceae and is the only documented nonaquatic CAM species.

It is postulated that CAM assists survival of *Z. zamiifolia* by reducing water loss and maintaining carbon gain during seasonal droughts characteristic of its natural habitat (Holtum et al. 2007).

Generally, in plants, intraspecific variations may be much evident in all aspects such as morphology, anatomy, cytology, palynology, biochemistry etc. Apart from genetic differences among individuals, much of the observed intraspecific variation is due to modifications during ontogeny. For example, depending on light conditions experienced during growth the leaves of a given individual may differ considerably in their photosynthetic response (Boardman 1977). There are many studies on photosynthetic rate of wild and cultivated crop species, like wheat (Evans et al. 1970), rice (Takano & Tsunoda 1971), barley (Chapin et al. 1989) etc. Most investigators reported that photosynthetic rate of some wild relatives of crop species was higher than that of the cultivated species (Cinthya Christopher 2000). In this backdrop, a comparative investigation on wild and cultivar of *Z. zamiifolia* on stomata and photosynthetic activity is significant and assumes importance. Hence, the present investigation is taken up to study the aspects in wild and cultivar of *Z. zamiifolia.*

MATERIALS AND METHODS

Field surveys were conducted to locate populations of *Z. zamiifolia* occurring in Kerala. During the survey, a wild and a cultivar of this species were collected and maintained in the Field Gene Bank of All Saints' College providing uniform environmental conditions. Stomatal characterization of leaf and leaflets were done. Five observations were scored for each character. Fresh mature leaves on 4th or 5th node from the shoot tip of the plants representing each accession, were collected and boiled in ethanol to remove the chlorophyll. The bleached leaves were kept overnight for incubation in 5% NaOH solution. The incubated leaves were washed in distilled water and the abaxial epidermis of the leaves peeled using forceps. The peeled epidermis was stained in 1% safranine, washed in distilled water, mounted in glycerine and observed under the microscope. The structure and types of stomata were determined as per the nomenclature and classification by Prabhakar (2004). The average value of the number of stomata, epidermal cells and sessile trichomes from 5 fields per leaf and 5 leaves per accession were calculated. Photomicrographs of the peels were taken using a Leica ICC50HD camera, attached to Leica DM 500 trinocular microscope. Slides as prepared for stomatal density are also used for determination of stomatal index by using the following formula:

$$\text{Stomatal index (\%)} = \frac{S}{S + E} \times 100$$

where, S and E are the number of stomata and epidermal cells respectively in microscopic field of view.

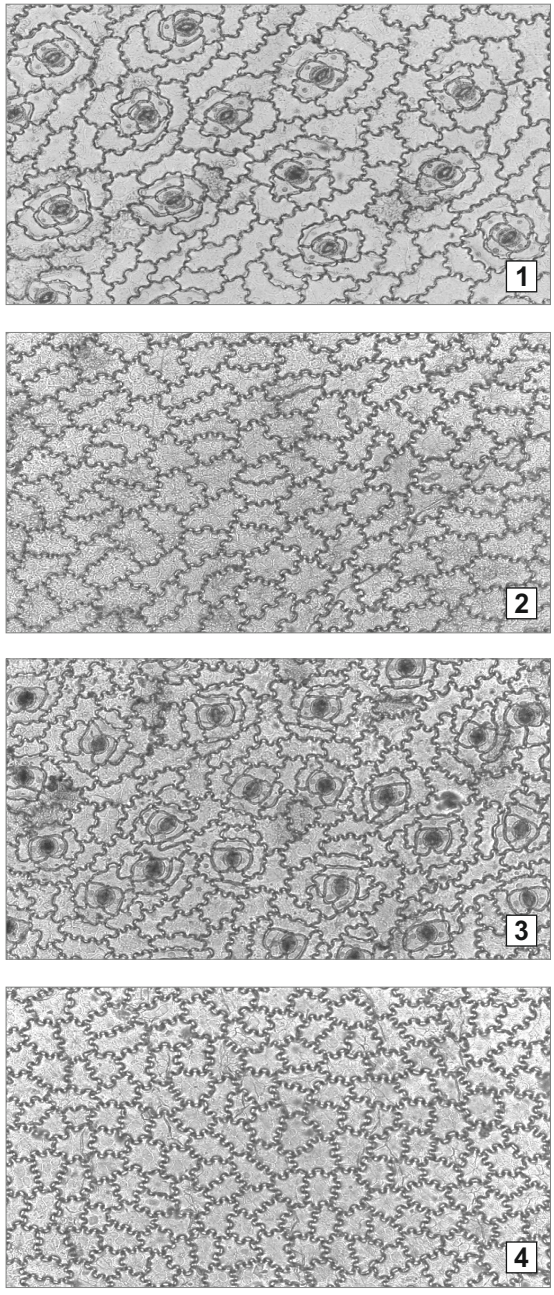
For the estimation of photosynthetic pigments, leaves are taken from the 5th node of the rachis. One g of fresh leaf tissue was taken and ground in 80% acetone. The ground mixture is

made up to 50 ml by filtering it in cheese cloth using acetone until the supernatant became colourless. Optical density was noted spectrophotometrically at different wavelengths, 420 nm, 440 nm, 470 nm, 490 nm, 520 nm, 540 nm, 570 nm, 600 nm, 630 nm, 643 nm, 645 nm, 652 nm, 663 nm, 670 nm, 690 nm and 720 nm. Estimation of the pigments were done as per Arnon (1949) based on extinction coefficient with respect to the solvent in which the extraction of pigment was done. Absorption spectra analysis was also done by constructing a graph plotted against wavelength against optical density.

OBSERVATIONS

Stomatal characteristics in wild and cultivar of *Z. zamiifolia* were studied. In both populations, stomatal type is paracytic with 2 subsidiary cells aligned parallel to the 2 guard cells and 2 cells arranged at the poles of the guard cells. Number of stomata per unit area, area occupied by the stomata in unit area, number of epidermal cells per unit area, area occupied by the epidermal cells in unit area and the stomatal index were calculated. The abaxial epidermal peel showed distinct stomata distributed throughout the epidermis with subsidiary cells. The epidermal cells are characterized by undulating margins. Both populations lack stomata on the adaxial surface.

The number of stomata per unit area (stomatal frequency) was 11.16 and 22.46 in wild and cultivar respectively (Figs 1–4). Even though the stomatal frequency was high for cultivar, the



Figs. 1–4: *Z. zamiifolia* 1. Lower epidermis of the wild plant. 2. Upper epidermis of the wild plant. 3. Lower epidermis of the cultivar. 4. Upper epidermis of the cultivar.

area occupied by the stomata in the wild was 0.09 mm^2 while that of the cultivar is only 0.05 mm^2. Frequency of epidermal cell in unit area was also higher for the cultivar (240.43) as compared to that of the wild (145.27). The area occupied by each epidermal cell in unit area was lesser for the cultivar (0.004mm^2) while that of the wild is higher (0.006 mm^2). Stomatal index of the wild and the cultivar was estimated to be 7.13 and 8.54 respectively. So, in the present study, it is evident that a strong negative correlation exists between the stomatal frequency and the area occupied by the stomata and also between frequency of epidermal cells and the area occupied by the epidermal cells.

Estimation of chlorophyll pigment in the wild and the cultivar of *Z. zamiifolia* showed that the total chlorophyll content was high in the wild, averaging 1.56 mg/g, as compared to that of the cultivar with only 1.01 mg/g. The same pattern was observed in the fraction of chlorophyll *a*, chlorophyll *b* and carotenoids, which were 1.004 mg/g, 0.55 mg/g and 3.45 mg/g in wild and in cultivar, the corresponding values are 0.65 mg/g, 0.36 mg/g and 2.18 mg/g respectively. But the ratio between chlorophyll *a* and *b* was high (1.803) for the cultivar while that of the wild was 1.79, difference being very insignificant. However, carotenoid content in the 2 populations of the species showed considerable variation. Whereas one showed 3.45 mg/g while the cultivar showed only 2.18 mg/g.

The chlorophyll pigments in the 2 populations were analyzed with the help of absorption spectra in the range of wavelength from 420 to 720 nm. The pigment extract of wild one showed an absorption maximum of the blue region at the wave length, 440 nm with a narrow fall at 520 nm. The peak absorbance at the red region represented the wavelength of 670 nm with a broad base between wavelengths of 652 and 690 nm. The pigment extract of the cultivar also showed an absorption maximum of the blue region at the wavelength of 440 nm with a narrow fall at 520 nm. The peak absorbance at the red region represented the wavelength of 670 nm with a broad base between wavelengths, 652 and 690 nm (Table 1, Fig. 5). The pattern of absorbency in different wavelengths of light in the 2 populations was the same. The absorption spectra of

TABLE 1: Absorbency of pigments noted in different wavelengths of light in wild and cultivar of *Z. zamiifolia.*

Wavelength	Optical Density	
	Wild	Cultivar
420	0.81	0.53
440	0.95	0.61
470	0.63	0.40
490	0.34	0.23
520	0.10	0.08
540	0.09	0.07
570	0.10	0.07
600	0.13	0.09
630	0.17	0.11
645	0.21	0.13
652	0.27	0.17
663	0.44	0.28
670	0.47	0.31
690	0.10	0.07
720	0.03	0.03

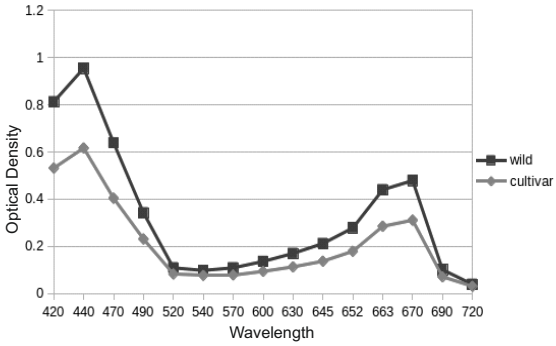


Fig. 5: Absorption spectra in wild and cultivar of *Z. zamiifolia.*

chlorophyll pigment in both the populations show that the wild one is more suited to the environment concerned than that of the cultivar, which is an evolved variety with low metabolic vitality.

DISCUSSION

Z. zamiifolia is grown as an ornamental plant, mainly for its attractive glossy foliage. The plant has air purifying qualities for the indoor environment. As an ancient plant, it has sustained substantial climate changes and attacks from millions of generations of pathogenic microorganisms (Moullec et al. 2015). Not only has it been described as a plant that is becoming or will become an important player in the foliage plant industry (Chen et al. 2002), but it was also listed among the Florida Plants of the Year in 2002. The ability of *Z. zamiifolia* to grow under low light condition, its tolerance to drought stress, its unique appearance, its low maintenance requirements and limited pest problems are characteristics that contribute significantly to its ornamental and landscaping value (Chen & Henny 2003).

The species has completely pinatisect leaves with unpaired pinnate leaflets in both wild and cultivar (subparipinnate). Leaf blade size is exceedingly diverse. The number of leaflets varies in the 2 variants studied. The distance between the leaflets also varies in the 2 variants. The wild has oblanceolate leaf whereas the shape of leaf in the cultivar is ovate. The average number of leaflets per rachis in the wild was 12 and that of the cultivar was 16. The petiolule is of much importance to the species as it is capable of regeneration. Plants are raised from the leaflets and the plantlets are developed from the leaf area with midrib. As the number of leaflets is higher and the arrangement of leaflets is more aesthetic, the cultivar is most preferred as indoor garden plant. The wild one is naturally adapted for propagation through leaflets as a small mechanical shock can remove the leaflet easily and regenerated easily.

The stomatal index was considered to be fairly constant within the leaves of a single species. Even though the stomatal number is determined by many extrinsic and intrinsic factors, the stomatal indices are quite constant and can be used in distinguishing different taxa (Poole et al. 1996). This matter was examined in the present material and found that the stomatal indices in the variants displayed striking variation. The stomatal indices of the wild and cultivar were 7.13 and 8.54 respectively. It is possible that this attribute may be genetically predisposed in the genotypes.

Photosynthetic capacity is closely linked to stomatal density (Xu & Zhou 2008). Moreover, photosynthetic potential might be enhanced with increased stomatal density in *Arabidopsis* by a modulating gas diffusion function, as reported by Tanaka et al. (2013). Recent studies (Zhao et al. 2015) had established significant negative correlations of stomatal density with photosynthesis and demonstrated that higher stomatal density reduced leaf photosynthesis. They argued that small stomata could maintain the pores opening with lower guard cell turgor pressures as compared to larger stomata. The higher stomatal density and reduced stomatal size responding to drought can effectively inhibit transpirative water loss and better water balance (Bosabalidis & Kofidis 2002).

It has been noted that in the wild *Z. zamiifolia,* even though the stomatal density and stomatal index are lower as compared to the cultivar, the photosynthetic efficiency may be high, which can be established by analyzing the chlorophyll content and absorption spectral analysis.

Estimation of chlorophyll pigment in wild and cultivar of *Z. zamiifolia* showed that the total chlorophyll content was high in the wild, averaging 1.56 mg/g, while in the cultivar the corresponding value was 1.01 mg g^{-1}. The same pattern was observed in the fraction of chlorophyll *a*, *b* and carotenoids, which were 1.004 mg/g, 0.55 mg/g and 3.45 mg/g in wild and in cultivar, 0.65 mg/g, 0.361mg/g and 2.189 mg/g respectively. But the ratio between chlorophyll *a* and *b* for the cultivar was 1.80 while that of the wild was 1.79, the difference being highly insignificant.

Carotenoid content in the wild and the cultivar showed considerable variation. Wild showed 3.458 mg/g while that of the cultivar was 2.189 mg/g. Thus the wild one is more benefited in this aspect as it showed an increase in the level of carotenoids.

The present study showed that the wild is photosynthetically more efficient than the cultivar and thereby indicating that the former is more active metabolically than the latter. It is in conformity with the earlier suggestion that wild plants have more vigour than the cultivars.

The chlorophyll pigments in the wild and cultivar were analyzed with the help of absorption spectra. The pigment extract of wild showed an absorption maximum of the blue region at the wavelength, 440 nm with a narrow fall at 520 nm. The peak absorbance at the red region represented the wavelength of 670 nm with a broad base between wavelengths 652 and 690 nm. The pigment extract of cultivar also showed an absorption maximum of the blue region at the wavelength, 440 nm with a narrow fall at 520 nm. The peak absorbance at the red region represented the wavelength 670 nm with a broad base between wavelengths 652 and 690 nm. The pattern of absorbency in different wavelengths of light in the 2 was the same (Fig. 5). Since the total chlorophyll, chlorophyll *a* and *b* were high in the wild, a relative increase in the quantity of the pigment was noticed in the absorption spectrum, as

evidenced by an increase in absorbency.

Recent improvements in the techniques of isolating and extracting plant pigments, primarily photosynthetic pigments, as well as identifying the biological reactions to which they may be coupled by action spectra and absorption spectra are useful in biochemical and/or physiological research (Buchanan 1980). From the present study on the absorption spectra of chlorophyll pigment of wild and cultivar of *Z. zamiifolia,* it is evident that the wild one is more suited to the environment concerned than that of the cultivar, which is an evolved cultivar variety with low metabolic vitality.

Further investigation is necessary on the stomatal conductance, gaseous exchange, and other parameters to substantiate the photosynthetic efficiency in the wild and the cultivar. However, both wild and cultivar of *Z. zamiifolia* can be used well as indoor plants as they use up the carbon dioxide and give out oxygen during the night since both are CAM plants.

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