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RESEARCH ARTICLE

IN VITRO REGENERATION AND MASS MULTIPLICATION OF AN ENDANGERED MEDICINAL PLANT *DECALEPIS ARAYALPATHRA*

MADHU MALLESHAPPA
School of Sciences, Department of Life Sciences, Garden City University, Bengaluru 560 049
For correspondence. Email: madhu.malleshappa@gardencity.university

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SUMMARY *Decalepis arayalpathra* (Joseph & Chandrasekaran) Venter**,** an endangered and endemic medicinal plant of Western Ghats was chosen for a large scale multiplication through direct regeneration. The nonembryogenic vegetative propagules such as apical and axillary buds from the field grown plants were used for the establishment of cultures. It was found that Murashigae and Skoog's Basal Medium (MSBM) supplemented with 6-benzylaminopurine (BAP) 2.5 mgl^{-1} and naphthalene acetic acid (NAA) 0.5 mgl^{-1} was found to be the most suitable for initiation, multiplication and root formation from the in vivo apical and axillary buds.

Keywords: *Decalepis arayalpathra*, endangered, medicinal, regeneration.

INTRODUCTION

Decalepis arayalpathra (Joseph & Chandrasekaran) Venter (Syn: *Janakia arayalpathra)* is a critically endangered and endemic medicinal plant belonging to family Periplocaceae. The latter was formerly included in the family Apocynaceae.

The tuberous roots of *D. arayalpathra* are highly aromatic and the native Kani tribes use it as an effective remedy against various diseases like peptic ulcer and cancer and as blood purifier and a rejuvenating tonic. (Pushpangadan 1990). Root extract of the plant revealed immuno-modulatory and anticancer properties. (Subramonian et al. 1996). The tubers are being ruthlessly collected from its natural habitat by the local Kani tribes. This has led to the acute scarcity of the plant. Consequently, it has been enlisted as an endangered plant species (CAMP-1 1995). The natural regeneration as well as conventional propagation of this plant is beset with several factors like poor fruit set, seed germination and rooting from stem cuttings (Sudha & Seeni 2001). Considering the urgent need for conservation, the present study has been undertaken to develop a repeatable protocol for large scale multiplication by in vitro technique for direct regeneration by using nonembryogenic propagules to produce genetically uniform plants on a large scale.

MATERIAL AND METHODS

D. arayalpathra was collected from the Western Ghats of Kerala, Bonacaud, (Latitude 8° 40' 36.2712" Longitude 77° 6' 5.1804") during 2004

and maintained in the Botanical Garden, Department of Botany, Bangalore University, Bengaluru..

The apical and axillary buds collected from field grown plants were used as explants for direct regeneration. They were washed thoroughly in tap water and with dilute solution of liquid detergent Labolene 5% (v/v) followed by rinsing several times in tap water to remove the traces of Labolene. They were further washed with distilled water for 4 or 5 times. The explants were brought onto Laminar Air Flow (LAF) unit, rinsed in 70% ethanol for 30 sec, disinfected in 0.1% mercuric chloride for 5 min and rinsed several times in sterile water to remove the traces of sterilant. The sterilized explants were inoculated on different nutrient media to study their morphogenetic response.

Murashige & Skoog's (1962) nutrient media was used along with the vitamins, glycine, nicotinic acid, pyridoxine HCl and thiamine HCl of cell culture tested grade from Sigma Chemicals, St. Louise, U.S.A. and auxins, α-Naphthalene acetic acid (NAA), 2, 4-Dichlorophenoxyacetic acid (2,4-D) and cytokinins, 6-Benzylaminopurine (BAP) and kinetin (Kn) obtained from, Hi-Media were used. The required quantities of auxins and cytokinins were dissolved in minimum quantity of ethyl alcohol (50%) and 1N NaOH respectively. Then, the required volume was made up with distilled water to prepare stock solutions. Sucrose was used at the rate of 3% (30 gl^{-1}) as a carbon source. Agar was used at the concentration of 0.8% (8 gl^{-1}) as gelling agent. Double distilled water (glass distillation) was used for the preparation of stock solutions, growth regulators, nutrient media, reagents and buffers.

The sterilized apical and axillary bud explants were cultured on Murashigae and Skoog's Basal Medium (MSBM) supplemented with BAP (2.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) respectively to obtain in vitro plantlets. All cultures were incubated at a temperature of 25 ± 2^{0} C under built-in white fluorescent light at a photon density of 30–50 $IEM^{-2}S^{-1}$ with 30–35% relative humidity (RH) under a photoperiod regime of 16 h light and 8 h dark cycles.

The in vitro shoots were subcultured on MSBM fortified with different concentrations of growth regulators to obtain multiple shoots. The cultures were scored for shoot initiation after 15 d of culture. Regeneration frequency (%) was computed as the number of explants responding to that of total number of explants inoculated.

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of cultures responding}}{\text{Total number of explants cultured}} \times 100$$

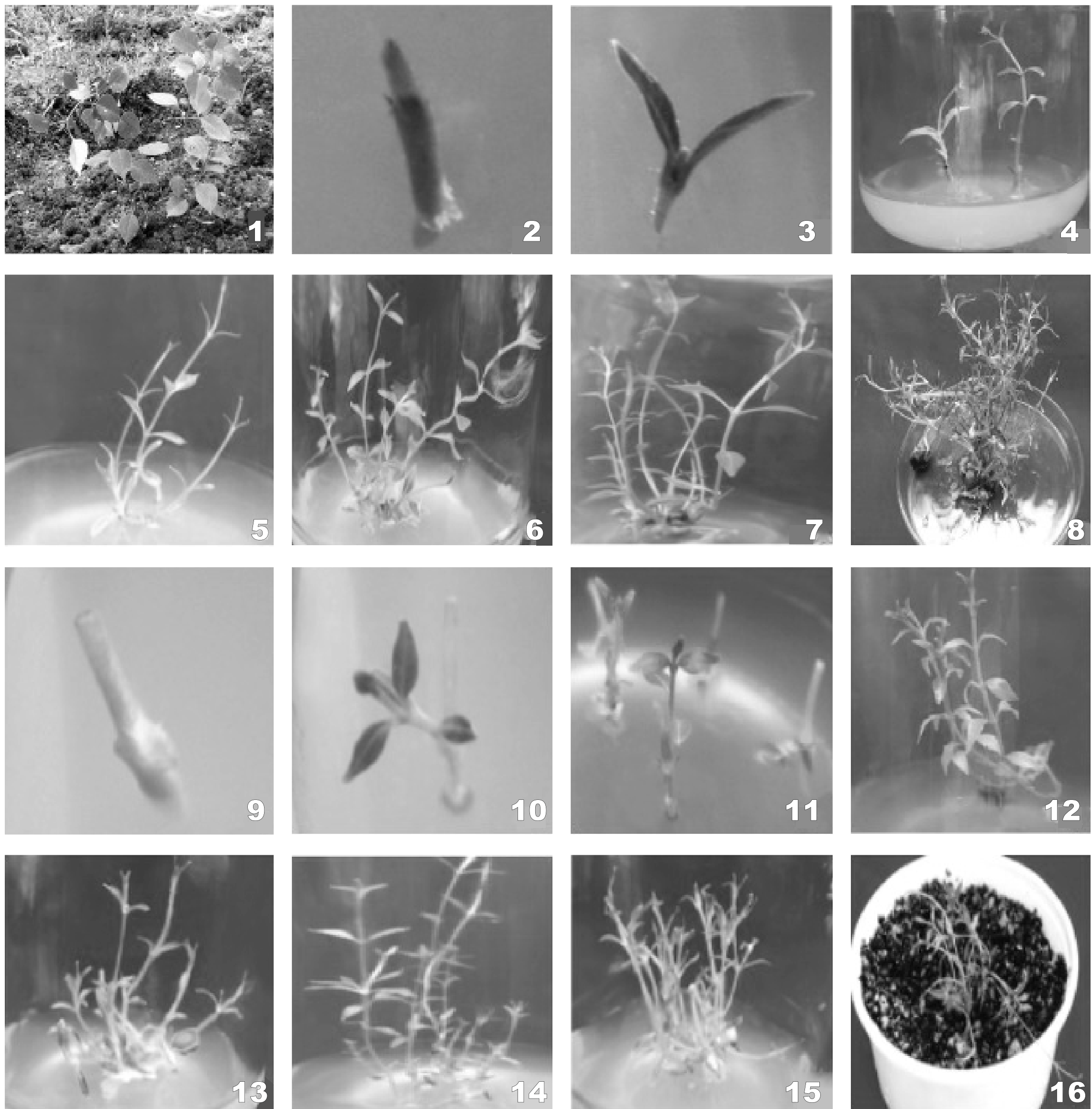
The well-developed plantlets were carefully taken out from the culture bottles and washed thoroughly with water to remove traces of agar. The plantlets were then transferred to plastic pots containing potting mixture peat:perlite: vermiculate in the ratio 1:1:1 (v/v). The potted plants were covered with polythene covers to maintain humidity. These plantlets were maintained at temperature of 25 ± 2° C and 90–95% RH. After 15 d, the covers were removed, and the plants were gradually exposed to less humid conditions.

OBSERVATIONS

The apical buds of the field grown plant measuring 0.6 cm were excised and cultured on MSBM supplemented with BAP and NAA in different concentrations, 1.0 mgl^{-1}, 1.5 mgl^{-1}, 2.0 mgl^{-1}, 2.5 mgl^{-1} , 3.0 mgl^{-1} and 0.1 mgl^{-1}, 0.2 mgl^{-1}, 0.3 mgl^{-1}, 0.4 mgl^{-1} , 0.5 mgl^{-1} to study their effect on apical bud multiplication (Figs 1, 2).

After 15 d of culture, first and second leaf formation was observed and by 25 d of culture, elongation of shoot was noticed (Figs 3, 4). After 40 d of culture, further growth and elongation of shoot was observed at all the concentrations of growth regulators tried with 30–85% response. The highest percentage of response (85%) was recorded on MSBM fortified with BAP (2.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) and the lowest percentage of response (30%) was recorded on MSBM supplemented with BAP (3.0 mgl^{-1}) and NAA (0.4 mgl^{-1}) (Table 1, Fig. 17). After 60 d of culture, development of multiple shoots (3 or 4) were observed (Fig. 5). After 85 d of subculture, clump of 5–7 multiple shoots were noticed which attain the height of 5–8 cm (Figs 6, 7). These multiple shoots rooted on the same medium thereby eliminating an additional step for in vitro rooting. After 125 d of culture, well developed multiple shoots with roots were observed (Fig. 8) on the same medium which are subjected to hardening process to study their survival frequency. The statistical data of different experiments were compiled. The analysis of the results revealed that the mean number of shoots per explant ranged from 0.80–9.35. It was observed that the cultures responded differently in different treatments. However, the highest mean number (9.35) was observed on MSBM supplemented with BAP (2.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) and lowest mean number (0.80) was noticed on MSBM supplemented with BAP (3.0 mgl^{-1}) and NAA (0.4 mgl^{-1}) (Table 1, Fig. 17). It was found that MSBM supplemented with BAP (2.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) was found to be the most suitable medium for initiation, multiplication and root formation from the in vivo apical bud of *D. arayalpathra*.

The axillary buds from in vivo plants of *D. arayalpathra* measuring 0.8 cm were excised and cultured on MSBM fortified with BAP and NAA in different concentrations of 1.0 mgl^{-1}, 1.5 mgl^{-1}, 2.0 mgl^{-1}, 2.5 mgl^{-1}, 3.0 mgl^{-1} and 0.1 mgl^{-1}, 0.2 mgl^{-1}, 0.3 mgl^{-1}, 0.4 mgl^{-1} , 0.5 mgl^{-1} to study their effect on multiplication (Fig. 9). After 20 d of culture, shoot bud initiation with 3 or 4 leaves were observed at all the concentrations of growth regulators studied with varying percentage of response (Figs 10, 11, 18, Table 2). The highest (80%) and the lowest percentage (35%) of response was observed on MSBM supplemented with BAP (2.5mgl^{-1}) and NAA (0.5 mgl^{-1}) and MSBM fortified with BAP (1.0mgl^{-1}) and NAA (0.1 mgl^{-1}) respectively. After 30 d of culture, the shoots were subcultured on the same medium to obtain more number of multiple shoots. After 40 d of subculture 3 or 4 multiple shoots were noticed on MSBM fortified with BAP (2.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) which formed a clump of 8–10 multiple shoots after 65 d of subculture (Figs. 12–14). The multiple shoots elongated and attain the length of 12–15 cm after 95 d of subculture (Fig. 15). The well developed shoots rooted on the same medium thereby eliminating an



Figs 1–16: *D. arayalpathra*. Apical bud and axillary bud explants on MSBM + BAP (2.5 mgl^{-1}) + NAA (0.5mgl^{-1}). 1. Field grown plant. 2. Apical bud explant at culture. 3. First and second leaf after 15 d of culture. 4. 25 d old culture showing elongation of shoots. 5. 60 d old culture showing multiple shoot formation. 6. Clump of (5–7) multiple shoots after 85 d of subculture. 7. Healthy elongated well-developed multiple shoots after 125 d of subculture. 8. Hardened in vitro plantlets. 9. Axillary bud explant at culture. 10,11. Shoot initiation with 3 or 4 leaves after 20 d of culture. 12. Formation of 2 or 3 multiple shoots after 30 d of culture. 13,14. Formation of 8–10 multiple shoots after 65 d of subculture. 15. Elongation of multiple shoot after 125 d of subculture. 16. Hardened in vitro plantlets.

TABLE 1: Effect of different concentrations of growth regulators for initiation and multiplication of shoots from apical bud explants of *D. arayalpathra* on MSBM.

BAP (mgl^{-1})	NAA (mgl^{-1})	Response (%)	No. of shoots/explant X*± SD
1.0	0.1	40	1.50 ± 0.67
1.5	0.1	50	2.00 ± 1.05
2.0	0.1	60	3.00± 0.56
2.5	0.1	55	2.10 ± 0.89
3.0	0.1	48	1.90 ± 0.69
1.0	0.2	50	2.00 ± 0.66
1.5	0.2	55	2.50 ± 1.21
2.0	0.2	62	3.00 ± 0.58
2.5	0.2	47	1.70 ± 0.75
3.0	0.2	40	1.20 ± 1.54
1.0	0.3	56	2.00 ± 0.65
1.5	0.3	59	2.80 ± 0.35
2.0	0.3	65	3.90 ± 1.58
2.5	0.3	50	2.10 ± 0.57
3.0	0.3	40	1.20 ± 0.29
1.0	0.4	45	1.40 ± 0.55
1.5	0.4	50	2.00 ± 0.46
2.0	0.4	58	2.70 ± 0.35
2.5	0.4	35	1.00 ± 0.43
3.0	0.4	30	0.80 ± 0.86
1.0	0.5	65	3.80 ± 1.58
1.5	0.5	70	4.80 ± 0.69
2.0	0.5	78	5.00 ± 1. 00
2.5	0.5	85	9.35 ± 1.59
3.0	0.5	68	3.90 ± 1.99

ANalysis of VAriance (ANOVA) TABLE (for No. of shoots /explant).

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F_{cal} ratio	F_{cal} value**	Critical difference
Treatments	24	764	31.83	39.78	1.54	0.61
Error	225	180	0.8			
Total	249	944				

*: Mean of 10 replications. **: Significant F value@5% level.

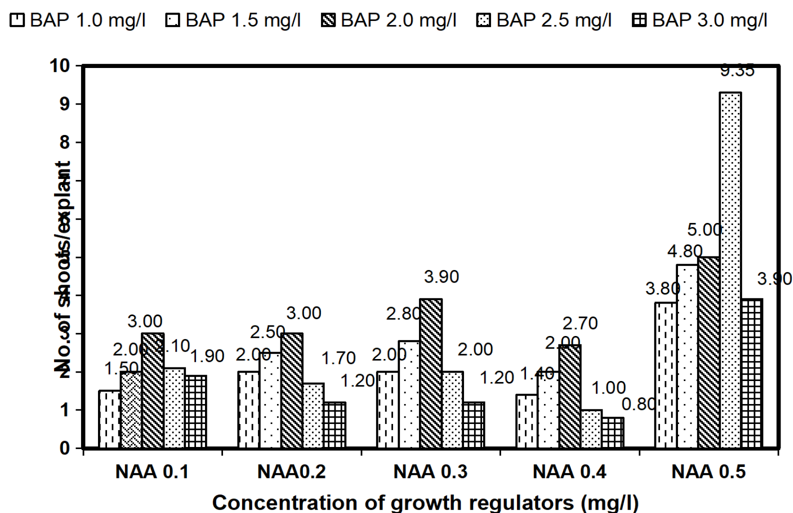
TABLE 2: Effect of different concentrations of growth regulators for initiation and multiplication of shoots from axillary bud explants of *D. arayalpathra* on MSBM.

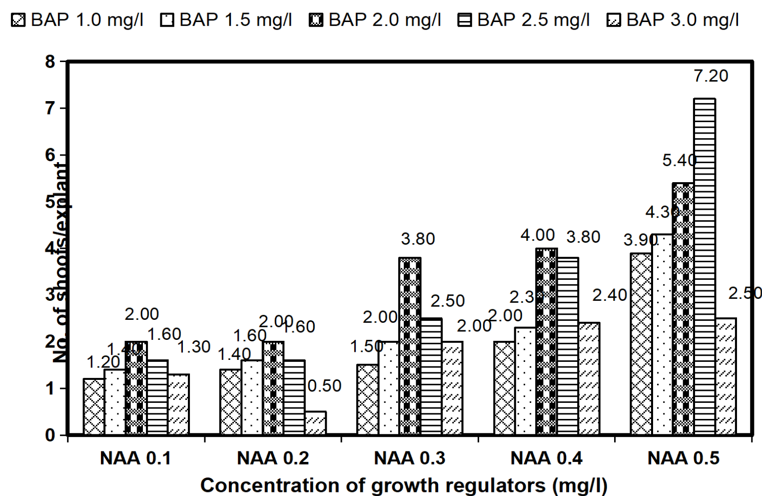
BAP (mgl^{-1})	NAA (mgl^{-1})	Response (%)	No. of shoots/explant X*± SD
1.0	**0.1**	**35**	**1.20 ± 0.24**
1.5	0.1	40	1.40 ± 0.52
2.0	0.1	50	2.00 ± 0.68
2.5	0.1	45	1.60 ± 0.23
3.0	0.1	41	1.30 ± 0.98
1.0	0.2	40	1.40 ± 1.21
1.5	0.2	45	1.60 ± 0.82
2.0	0.2	50	2.00 ± 1.21
2.5	0.2	45	1.60 ± 0.67
3.0	0.2	39	0.50 ± 0.88
1.0	0.3	45	1.50 ± 0.34
1.5	0.3	49	2.00 ± 0.22
2.0	0.3	62	3.80 ± 0.48
2.5	0.3	58	2.50 ± 0.66
3.0	0.3	50	2.00 ± 1.41
1.0	0.4	48	2.00 ± 0.12
1.5	0.4	52	2.30 ± 0.53
2.0	0.4	65	4.00 ± 0.11
2.5	0.4	60	3.80 ± 0.23
3.0	0.4	53	2.40 ± 0.72
1.0	0.5	60	3.90 ± 0.87
1.5	0.5	70	4.30 ± 0.72
2.0	0.5	76	5.40 ± 1.21
2.5	**0.5**	**80**	**7.20 ± 1.87**
3.0	0.5	58	2.50 ± 0.67

ANalysis Of VAriance (ANOVA) TABLE (for No. of shoots/explant).

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F_{cal} ratio	F_{tab} value**	Critical difference
Treatments	24	764	31.83	**39.78**	**1.54**	**0.61**
Error	225	180	0.8			
Total	249	944				

*: Mean of 10 replications. **: Significant F value@5% level.





Figs 17&18: *D. arayalpathra.* Effect of different concentrations of growth regulators on multiple shoot formation from apical and axillary bud explants.

additional in vitro step for rooting after 125 d of culture. The rooted axenic plants were subjected to hardening process to study their survival frequency (Fig. 16). In the present investigation, the statistical analysis of the data revealed that there exists a highly significant difference between and within the treatments. The mean number of shoots per explant ranged from 1.20–7.20 (Table 2, Fig. 18). The highest mean number (7.20) was observed on MSBM supplemented with BAP (2.5 mgl^{-1}) and NAA (0.5mgl^{-1}) and the lowest mean number (1.2) was noticed on MSBM fortified with BAP (1.0 mgl^{-1}) and NAA(0.1mgl^{-1}).

It was observed that MSBM supplemented with BAP (2.5mgl^{-1}) and NAA (0.5mgl^{-1}) was found to be the best medium for axillary bud initiation, multiplication and root formation from axenic plants.

DISCUSSION

D. arayalpathra is generally propagated by seeds and vegetative cuttings. However, natural regeneration as well as conventional breeding programme in this plant are not possible due to lack of natural seed cycle. Therefore, it is imperative to develop efficient in vitro protocols using nonembryogenic vegetative propagules from different parts of the plant for micro-propagation through direct regeneration and also for long-term conservation by synthetic seeds. MSBM supplemented with BAP (2.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) was found to be the best medium for apical bud multiplication of *D. arayalpathra* with highest mean number of 9.35 multiple shoots per culture. This agrees with the findings of Sarkar et al. (1996) and Ghosh & Banerjee (2003). They reported that BAP (5.0 mgl^{-1}) and NAA (1.0 mgl^{-1}) are essential for induction of multiple shoots. Further, in the present study, it was found that the combination of auxin and cytokinin is essential for apical bud multi-plication. This concurs with the findings of Ghosh & Banerjee (2003).

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RESEARCH ARTICLE

PHYTOCHEMICAL, ANTIBACTERIAL AND SPECTROSCOPIC ANALYSES OF *CHRISTELLA DENTATA*

A. P. THOMAS[1*], N. SINDU[1], S. DILEESH [2], IYNTHAVY KRISHNA[1] AND P. M. MATHEW[3**]
[1] Department of Botany, St. Peter's College, Kolenchery 682 311, India
[2] Department of Chemistry, St. Peter's College, Kolenchery 682 311, India
[3] Perakathuseril, Muttada P. O., Thiruvananthapuram 695 025, India
*For correspondence. Email: amosepthomas@gmail.com

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SUMMARY Phytochemical, antibacterial and FT-IR spectroscopic analyses of *Christella dentata* (Forssk.) Brownsey & Jermy, commonly called the 'tapering tri-vein fern' was carried out. Fronds were extracted by hot solvent extraction method with soxhlet apparatus using petroleum ether, ethyl acetate, methanol and water. Phytochemical evaluation of the extracts revealed the presence of various secondary metabolites such as alkaloids, cardioglycosides, phenolics, saponins, tannins, terpenoids, steroids, quinones, flavonoids, glycosides, anthocyanins and betacyanins. Extracts were subjected to antibacterial assay using disc diffusion method, against four pathogenic bacterial strains such as *Klebsiella pneumoniae, Salmonella typhimurium, Escherichia coli* and *Xanthomonas campestris*. The results showed that ethyl acetate and methanolic extracts were active whereas petroleum ether and aqueous extracts were inactive. The peak values and chemical groups in FT-IR spectroscopic analysis confirmed the presence of various phytochemical compounds.

Keywords: *Christella dentata,* Thelypteridaceae, antibacterial, phytochemical, FT-IR.

INTRODUCTION

Pteridophytes provide an important contribution to the earth's plant diversity. Being the second largest group of vascular plants, they hold a significant position in many plant communities. Unfortunately, the medicinal and economic importance of ferns is ignored as compared to higher plant groups (Nair 1959). The known studies are those of Nath et al. (2016) reporting the antibacterial activity of some ethnobotanically important ferns of Southern Assam such as *Adiantum capillus-veneris, Asplenium nidus, Cyathea brunoniana, C. gigantea, Dipteris wallichi, Drynaria quercifolia, Lygodium japonicum, L. flexuosum, Pityrogramma calomelanos* and *Pteris biaurita* against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Proteus vulgaris*; Paul et al. (2011) analysed the phytochemical and antibacterial activity of

** Formerly Professor and Head of the Department of Botany, University of Kerala, Kariavattom.

Christella parasitica and found epidermal glands, which act as the key factors for intraspecific variation in antibacterial activity; the phytochemical studies carried out by Muraleedharannair et al. (2012) on extracts of *Adiantum caudataum, A. latifolium, A. lunulatum, Christella dentata* and *C. parasitica* revealed the presence of carbohydrates, steroids, tannins, saponins, carboxylic acid, coumarins, xanthoprotein and phenolic compounds. The phytochemical analysis, antimicrobial activity and identification of phytoconstituents in *Gleichenia pectinata* by Femi-Adepoju et al. (2018) showed the inhibitory activity against *P. aeruginosa* and *Klebsiella pneumoniae*.

Christella dentata belongs to the Class Polypodiopsida, Order Polypodiales and Family Thelypteridaceae. The present work is aimed at analysing and evaluating the preliminary phytochemical screening, antibacterial properties and FT-IR spectroscopic analysis of the frond extracts of *Christella dentata* (Forssk.) Brownsey & Jermy.

MATERIAL AND METHODS

Preparation of extracts

Specimens for the present study were collected from Nedumkandam, Idukki District, Kerala and authenticated by Dr. Raju Antony of Jawarharlal Nehru Tropical Botanical Garden & Research Institute, Palode, Thiruvananthapuram and deposited in the herbarium of Kerala Forest Research Institute, Peechi, Thrissur (No. 18099). The fronds were washed thoroughly, shade dried and powdered with a blender. Extracts were prepared using soxhlet extractor. 30 g of powdered samples were extracted successively with 150 ml of petroleum ether, ethyl acetate, methanol and distilled water for 8–12 h at a temperature not exceeding the boiling point. The extracts were concentrated in vacuum using a rotary evaporator.

Qualitative phytochemical screening

Different qualitative chemical tests were performed on various extracts to detect the presence of phytoconstituents (Harborne 1998).

For testing alkaloids, 1 ml of the plant extract was mixed with 1 ml of 1% HCl, warmed and filtered. 2 ml of filtrate was treated with Mayer's reagent. Turbidity or precipitation, green colour indicates the presence of alkaloid. For testing cardioglycosides, 1 ml of the extract was mixed with 2 ml of glacial acetic acid to which added a few drops of 5% ferric chloride. This was under layered with 1 ml of concentrated sulphuric acid. Formation of a brown ring at the interface indicates the presence of cardioglycosides. To test phenolic compounds, 1 ml of extract was mixed with 2 ml of distilled water, 0.5 ml of sodium carbonate and Folin ciocalteau's reagent. Formation of a blue or green colour indicates the presence of phenol. For testing saponins, 1 ml of plant extract was dissolved in 2 ml of boiling water in a boiling tube, allowed to cool and shaken well to mix. The appearance of foam

indicates the presence of saponins. To detect tannins, 2 ml of the test solution was mixed with 2 ml of ferric chloride. The formation of a blue-black or dark green colour indicates the presence of tannins. For testing terpenoids, 1 ml of extract was mixed with 2 ml of chloroform and 1.5 ml of concentrated sulphuric acid. Formation of a reddish brown colour indicates the presence of terpenoids. For testing steroids, 1 ml of plant extract was mixed with 2 ml of chloroform and 1 ml of sulphuric acid. Formation of a reddish brown colour at the interface indicates the presence of steroids. To detect quinines, 1 ml of extract was mixed with 1 ml of concentrated sulphuric acid. Formation of red colour indicates the presence of quinones. For testing flavonoids, 3 ml of extract was mixed with 4 ml of 1N sodium hydroxide. Formation of dark yellow colour indicates the presence of flavonoids. For testing glycosides, 2 ml of extract was mixed with 3 ml of chloroform and 1 ml of 10% ammonium solution. Formation of pink colour indicates the presence of glycosides. To detect anthocyanins and betacyanins, 1 ml of 2N NaOH was added to 2 ml of extract. This mixture was allowed to heat for 5 min at 100^0 C. Formation of bluish green colour indicates the presence of anthocyanins, whereas yellow colour indicates the presence of betacyanins.

Test for antibacterial activity

The agar disc diffusion method was used to evaluate the antibacterial activity (Murray et al. 1957). The antibacterial activity of different extracts was tested on pathogenic bacteria such as *Klebsiella pneumoniae* (NCIM 2883) *Salmonella typhimurium* (NCIM 2501), *Escherichia coli* (NCIM 5846) and *Xanthomonas campestris* (NCIM 5028), obtained from the CSIR National Chemical Laboratory, Pune. Standard antibiotic discs such as Amoxyclav (30 mcg) and Methicillin (5 mcg) were used as the positive control. Solvents like petroleum ether, ethyl acetate, methanol and distilled water were used as the negative control. The test for antibacterial activity was carried out by measuring the diameter of inhibition zone (in mm). The experiment was repeated thrice and the results were the mean of three replicates.

FT-IR spectroscopic analysis

Extracts were subjected to FT-IR spectroscopic analysis for detecting the functional groups present. The characteristic peaks were determined and their functional groups were analysed using Fourier-transform infrared spectrophotometer (Shimadzu) with a scan ranging from 600 to 4400 cm^{-1} and a resolution of 4 cm^{-1}. Peak values were recorded and functional groups were analysed (Sahaya et al. 2012).

OBSERVATIONS

Qualitative phytochemical analysis

Preliminary phytochemical analysis carried out in different extracts of *C. dentata* indicated the presence of various secondary metabolites. Extracts of fronds showed the presence of phytoconstituents such as alkaloids, cardio-

glycosides, phenols, saponins, tannins, terpenoids, steroids, quinones, flavonoids, glycosides, anthocyanins and betacyanins (Table 1). Ethyl acetate and methanol extracts yielded more secondary metabolites than petroleum ether and aqueous extracts. Most of the phytoconstituents showed strongly positive results in methanol extracts.

TABLE 1: Phytochemical analysis of *C. dentata*.

Phytoconstituent	Extract			
	PE	EA	M	A
Alkaloid	++	+++	+	-
Cardioglycoside	++	++	+++	-
Phenol	-	++	++	++
Saponin	++	+	+++	+++
Tannin	++	++	+++	+++
Terpenoid	+	+	+++	++
Steroid	+	++	++	-
Quinone	++	++	+++	++
Flavonoid	+	+++	++	++
Glycoside	+++	+	+++	-
Anthocyanin	++	+++	-	-
Betacyanin	+++	++	+	-

PE, Petroleum ether; EA, Ethyl acetate; M, Methanol; A, Water. **+++**, Strongly positive; **++**, Moderately positive; **+**, Positive; -, Negative.

Antibacterial evaluation

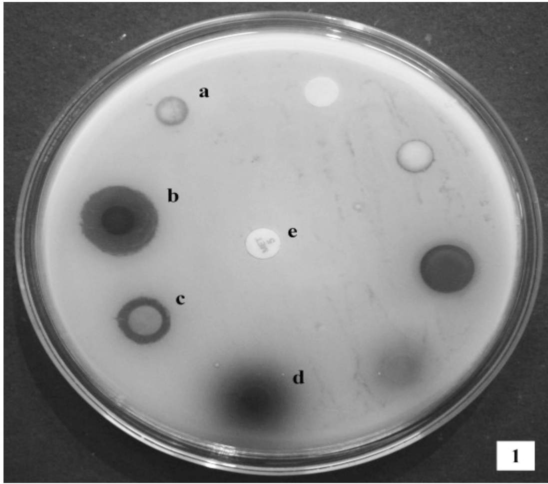
The different solvent extracts of *C. dentata* fronds were found to be effective against all the bacterial strains tested. Ethyl acetate and methanolic extracts of fronds showed significant antibacterial activity aganist *K. pneumoniae, S. typhimurium, E. coli* and *X. campestris* (Table 2).

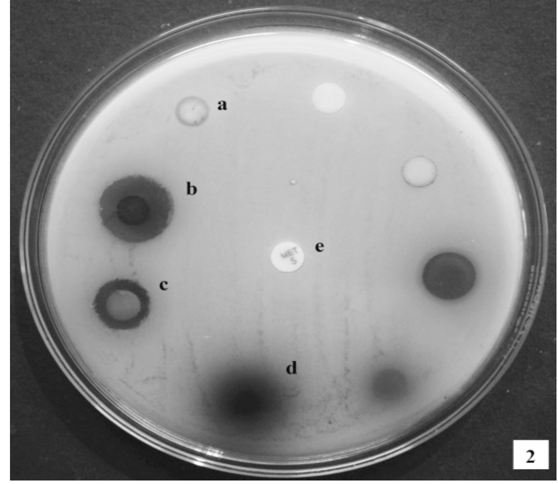
TABLE 2: Antibacterial evaluation of *C. dentata.*

Zone of inhibition (mm): Values = Mean ± S. D										
Bacterial strain	Extract				Negative control				Positive control	
	PE	EA	M	A	PE	EA	M	A	Methicillin (5 mcg)	Amoxyclav (30 mcg)
K.pneumoniae	0	10.33 ± 0.57	9.33 ± 0.57	0	0	0	0	0	0	0
S.typhimurium	0	15 ± 1	11.16 ± 1.04	0	0	0	0	0	0	10 ± 0
E. coli	0	12.83 ± 0.76	13 ± 1	0	0	0	0	0	0	8.5 ± 0.57
X. campestris	0	16 ± 1	10.5 ± 0.5	0	0	0	0	0	0	9 ± 1

S.D, Standard deviation; PE, Petroleum ether; EA, Ethyl acetate; M, Methanol; A, Water.

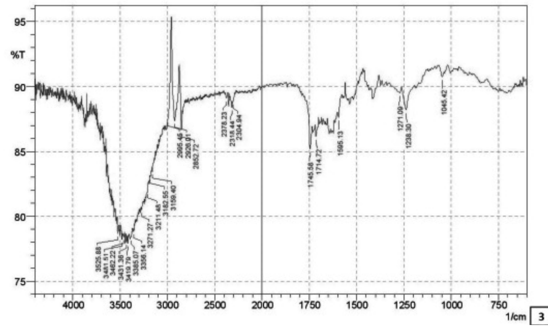
Ethyl acetate and methanol extract of fronds were sensitive to every bacterial strains tested whereas, petroleum ether and aqueous extracts were not sensitive. While comparing the effect of ethyl acetate and methanol extracts, the extract in ethyl acetate showed considerable inhibition on bacterial growth. The respective pure solvents that were taken as negative control showed no inhibitory action. The antibiotic, Amoxyclav (30 mcg) showed zone of inhibition against the bacterial strains tested whereas Methicillin (5 mcg) did not show any response (Figs 1, 2). The

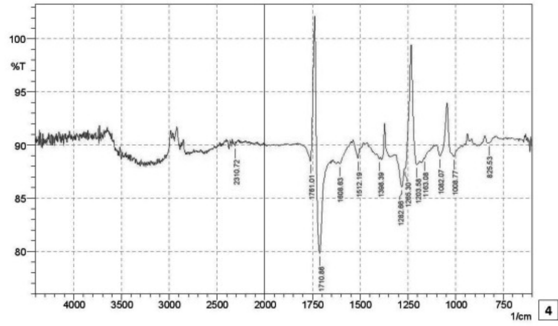


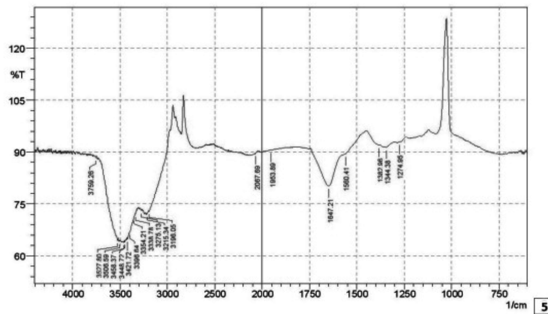


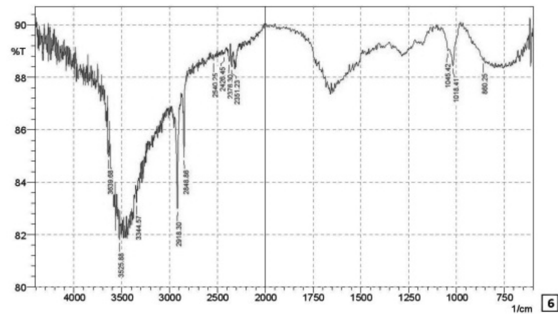
Figs 1–2: Antibacterial activity of *C. dentata* frond. 1. Against *S. Typhimurium.* 2. Against *X. campestris* (a, Petroleum ether extract; b, Ethyl acetate extract; c, Methanol extract; d, Aqueous extract; e, Methicillin)

FT-IR spectrum of *C. dentata* revealed the existence of various active components in different extracts (Figs 3–6). The results confirmed the presence of alkyl amines, nitro compounds, aromatics, ketones, saturated aliphatics, phosphines, alkanes, carboxylic acid,









Figs 3–6: FT-IR spectrum of *C. dentata* frond extracts. 1. Petroleum ether. 2. Ethyl acetate. 3. Methanol. 4. Aqueous.

alcohol, phenol, alkyl halides, esters, acid chloride, amine and aldehyde (Table 3).

TABLE 3: FT-IR spectroscopic evaluation of *C. dentata.*

Extract	Peak value	Functional group
Petroleum ether	1045.42	Alkyl amines
	1271.09	Nitro compounds
	1595.13	Aromatics
	1714.72	Ketones , saturated aliphatics
	1745.58	Esters, saturated aliphatic compounds
	2318.44	Phosphines
	2926.01	Alkanes
	3182.55	Carboxylic acid
	3481	Alcohol, phenol
Ethyl acetate	825.53	Alkyl halides
	1203.58	Esters
	1282.66	Carboxylic acid
	1398.39	Alkane
	1512.19	Aromatic
	1761.01	Acid chloride
Methanol	1560.41	Amine
	1647.21	Alkanes
	3448.72	Alcohol
Aqueous	1018.41	Alcohol
	2376.30	Carboxylic acid
	2848.86	Aldehyde
	2918.30	Alkanes
	3525.88	Alcohol

DISCUSSION

The ablility to synthesize secondary metabolites with antimicrobial capacity makes plants invaluable in the combat against recently observed resistance of microorganisms to antibiotics (Lis-Balchin & Deans 1997). The attribute of antimicrobial activity may be due to the presence of one or more bioactive compounds such as alkaloids, steroids, saponins etc. (Balandrin & Klocke 1988). Thomas et al. (2020) carried out the comparative phytochemical and antibacterial analyses of rhizome and frond extracts of *Pityrogramma calomelanos* and reported the presence of various secondary metabolites such as alkaloids, cardioglycosides, phenolics, saponins, tannins, terpenoids, steroids, quinones, flavonoids and glycosides. Extracts of fronds exhibited significant antibacterial activity as compared to rhizome extracts against *S. typhimurium*, *P. aeruginosa*, *E. coli* and *X. campestris*. The present study also revealed the presence of various phytoconstituents in *C. dentata*.

Previous studies revealed that *C. dentata* possesses antifungal and antibacterial properties. The presence of antimicrobial activity of this species may be due to the presence of one or more bioactive compounds such as alkaloids, glycosides, flavonoids, steroids, saponins etc. The methanol extracts of fronds were sensitive to *Rhodococcus pyrinidivorans* and insensitive to *Geobacillus stearothermophilus* (Manhas et al. 2018). *Tectaria coadunata* and *T. wightii* of order Polypodiales also possesss antibacterial efficacy against *K. pneumoniae, S. typhimurium, P. aeruginosa, E. coli, Listeria monocytogenes* and *X. campestris*. Ethyl acetate and methanol extracts of fronds were more efficient rather than the extracts of rhizome (Thomas et al. 2021).

Similarly, the present study is suggestive that the extracts of *C. dentata* are sensitive to *K. pneumoniae, S. typhimurium, E. coli* and *X. campestris*. The result profile of FT-IR spectrum analysis of *Phymatosorus scolopendria* determined various active components in ethanol extracts such as amines, alkanes, alkynes, alkenes, ether and alkyl halides (Sujatha et al. 2018). The present findings also confirm the presence of various functional groups and secondary metabolites in *C. dentata.*

The current findings have indicated the antibacterial activity of the ethyl acetate and methanolic extracts of *C. denatata* fronds as very effective against all the four bacterial strains tested because of the various phytochemicals present. Qualitative phytochemical screening and FT-IR spectroscopic analysis had confirmed the existence of secondary metabolites in this species. This study concurs with the earlier findings that ferns should be further screened for bioactive compounds. *C. dentata* having several phytochemicals owes remarkable antibacterial efficacy.

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RESEARCH ARTICLE

WAY TOWARDS BREEDING ONION FOR BETTER STORAGE LIFE

B. B. MADALAGERI*, PRAVEEN NOOJIBAIL, M. G. PURUSHOTHAMA, G. SHIVAKUMAR AND D. JEEVITHA

Department of Vegetable Breeding and Department of Biotechnology, I & B Seeds Private Limited, Bengaluru 560 060

*For correspondence. Email: madalageribb@gmail.com

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SUMMARY Series of experiments on breeding for minimizing storage losses of onion were conducted during 2016–2021. Germplasm evaluation indicated that yellow onions were damaged to an extent of 60% in extended period of storage. Genetic parameters showed high percentage of heritability for storage losses but genetic advance was low. F_1 breeding was better than polycrosses or open pollinated varieties in onion storage, and the best hybrid was RH8 with good stability indices and least losses. Selecting bulbs in the segregating population and advancing the population to further generations (F_6 to F_7) provided futuristic good storage type onions.

Keywords: Onion, *Allium cepa*, storage, genetics, breeding.

INTRODUCTION

Although India produces 19.4 million metric tonnes of onion, only 40–50% of it reaches to consumers because of various types of post-harvest losses like physiological loss in weight (about 23%), sprouting loss (4–5%) and rotting loss (50%) (Adamicki 2005, Tripathi & Lawande 2006,). The losses can be minimized if there are varieties/hybrids with better storage ability (Tripathi 2008), genetic factors (Abu-Goukh et al. 2001), cultural practices, seasons of cultivation (Srivatsava et al. 1996), pre- and post-harvest treatments before storing and storage facilities and atmosphere etc. (Brice et al. 1997, Skultab &Thompson 1992).

In India, with tropical climatic conditions, onions are grown almost throughout the year and fresh arrivals to market are at every 4 month (M) intervals. Therefore, the harvested onions need to be kept unspoiled for at least 4 M in storage until the next harvest season. Onion growing farmers in India (especially in Karnataka, Maharashtra, Madhya Pradesh, Odisha and Tamil Nadu) do not have storage facilities to store onions in unspoiled condition. Therefore, an attempt is made here to identify the onion variety or to breed good storage type onion variety or hybrid by different breeding methods as suggested by Madalageri (1983) on the basis of genetic factors controlling onion storage characters.

MATERIAL AND METHODS

The available onion germplasms in the gene bank of I & B Seeds, Bengaluru were raised for bulb production in *kharif* and *winter* season of 2016 and stored in perforated nylon bags under room temperature (25–35° C). Observations on weight loss, loss due to sprouting and rotting were recorded at 1 M and 4 M after storage. Genetic parameters were worked out.

In the following year, 5 polycross hybrids (PCH) were developed using the best performing genotypes by planting the bulbs in separate rows and allowing them for random crossing by bees under natural condition. The seeds were collected from each row and tested their performance in different seasons (*kharif, rabi* and *summer*) along with checks by raising the bulbs in Randomized Block Design (RBD) and bulbs were again stored under room temperature.

Knowing the performance of hybrids being better than commercial varieties from our experiments, elaborate hybridization programme was initiated using selected inbred lines in the year 2018 and hybrids of red, white and yellow were developed on respective Cytoplasmic Male Sterile (CMS) lines. They were tested again in all the 3 seasons and their bulbs were subjected to storage up to 4 M.

The heterosis was worked out on mid parent (MP), better parent (BP), best parent and commercial check on 4 M storage of *kharif* season bulbs. Further, using 3 seasons' data ranking of hybrids on per se values and stability was worked out following Eberhart & Russel's model (1966). For this purpose, the per se values of per cent total weight loss was converted to log values as there were many values with 100% loss.

In yet another attempt, doubled haploid (DH) breeding method was attempted by using few dark red, light red, white and yellow genotypes in the company's biotechnology laboratory following the protocol as described by Martinez et al. (2000). Only red genotypes responded while white and yellow did not. The DHs so developed were allowed to bulb in the green house and these bulbs were used to produce viable seeds in the winter of 2019. Only 2 DH lines produced viable seeds. These lines were sown along with commercial checks in *kharif* season of 2020. Bulbs were stored as indicated and their storage losses were recorded. DH lines were also included in hybridization programme to test their utility as parents.

In a separate experiment, white male sterile line was crossed with dark red line and yellow line to get F_1s. The hybrids were raised with checks to study their storage performance. The bulbs of F_1s were planted after storage for seed production to get F_2 seeds which were resown to study the segregation pattern and the segregants were stored as usual and analyzed the possibilities of selecting the bulbs for better storage types. Further, the generations were advanced up to F_4.

OBSERVATIONS

The total storage losses (physiological weight loss, losses due to sprouting and rotting) in 1 M and 4 M storage of different coloured onion bulbs averaged over 2 seasons is presented in Fig. 1.

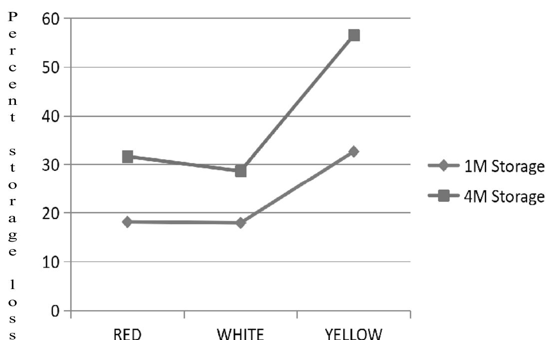


Fig. 1: Behaviour of different coloured onions in storage.

Red and white onions exhibited least loss (about 20%) as against yellow onions (about 32%) in 1 M holding and similar pattern was noticed in 4 M storage with additional 10% loss in red and white as against nearly 60% in yellow types.

Different genetic parameters suggest that the heritability (%) for storage losses was high enough (ranging from 52.75 to 71.44%) but genetic advance was low in all the cases (Table 1). There were genotypes with as low as 1% loss (16/43 LR) in 1 M to only 10% even after 4 M of storage (16/50 W). There were more number (8) of light red genotypes than dark red or white or yellow genotypes storing better than the rest of the genotypes.

TABLE 1: Genetic parameters for storage loss in onion genotypes.

	Kharif	*Kharif*	*Winter*	*Winter*
Months of storage	1 M	4 M	1 M	4 M
No. of genotypes	66	59	64	64
Genotypic variance	1.25	6.26	4.31	5.27
Phenotypic variance	2.36	8.76	6.38	7.56
Heritability (%)	52.75	71.44	67.50	69.70
Genetic advance	1.75	4.55	3.67	4.13
t -test	0.19(NS)	0.04(NS)	0.07(NS)	0.09(NS)
Genotypes with least loss	16/43 LR (1%)	16/15 LR (15%) 16/14 DR (15%)	16/10 DR (6%) 16/39 DR (6%)	16/50 W (10%)
	16/16 LR (2%)	16/22 LR (16%)	16/42 DR (7%) 16/15 LR (7%)	16/15 LR (12%)
	16/10 DR(3.9%)	16/16 W (17%)	16/7 Y (8%) 16/67 W (8%) 16/43 LR (8%)	16/44 LR (13%)

LR, Light red types; DR, Dark red types; W, White types; Y, Yellow.

Breeding methods were compared as to whether it is possible to develop storage type onions. The mean (52.69%) of F_1 hybrids was least as compared to polycross breeding and open pollinated varieties (OP) (62.42%) (Table 2).Therefore, many F_1 hybrids were developed and tested (Table 3). The heterotic hybrids (with negative values) indicated as good storers and per cent heterosis was worked out on MP, BP and best parent of the lot and over commercial check (Virat). There were respectively a total of 21, 13, 1 and 15 hybrids indicated good storage with negative heterosis. Out of those, there were 13, 8, 1 and 10 in red; 4, 3, 0 and 1 in white; and 4, 2, 0 and 4 in yellow hybrids showing negative heterosis. There was only one hybrid, RH8 that had negative heterosis over all component tests. The mean over hybrids on MP and check were negative (-5.69 and -5.60) while other 2 components were positive. The positive and negative values indicate that the character is controlled by both additive and dominant genes.

The ranking of hybrids on per se values with least storage loss in each season and on overall mean suggest that it was only RH8 that ranked in all cases with least overall mean values (49.36%)

TABLE 2: Performance of poly cross hybrids (PCH), F_1 hybrids and commercial checks in per cent loss of bulbs in 1 M storage.

Sl. No.	Genotype	*Kharif*	*Winter*	*Summer*	Overall mean (%)
	Polycross hybrids				
1	PCH 2	87.90	27.33	60.00	58.41
2	PCH 3	77.04	25.33	52.00	51.45
3	PCH 4	97.59	28.00	54.33	59.97
4	PCH 5	92.40	25.33	46.33	54.68
5	PCH 6	80.83	23.33	61.28	55.15
	Mean	87.15	25.86	54.78	55.93
	F_1 Hybrids				
1	Hybrid 1	98.82	26.00	26.00	50.27
2	Hybrid 2	100.00	29.66	35.66	55.11
	Mean	99.41	27.83	30.83	52.69
	Commercial checks				
1	16/20	96.59	34.33	64.00	64.97
2	16/26	92.26	27.66	59.66	59.86
	Mean	94.42	30.99	61.83	62.42
	SEM ±	8.83	7.01	15.4	5.44
	CV %	16.91	44.26	52.3	16.64
	CD @5%	NS	NS	NS	NS

TABLE 3: Heterosis in hybrids for storage losses (%) in 4 M of storage.

Sl. No.	Hybrid	% Heterosis			
		MP	BP	Best P	Check (Virat)
1	RH1	-6.57	7.51	49.27	-11.96
2	RH2	-14.29	7.95	28.42	-24.26
3	RH3	15.02	53.01	66.53	-1.78
4	RH4	-32.21	-28.13	15.92	-31.63
5	RH5	-11.33	24.45	24.45	-26.60
6	RH6	-6.42	-4.43	72.70	1.85
7	RH7	-4.97	-2.95	75.39	3.43
8	RH8	-47.67	-39.48	-16.70	-50.87
9	RH9	-2.04	0.033	80.78	6.61
10	RH10	-16.14	-14.35	54.77	-8.72
11	RH11	-5.60	-3.59	74.22	2.74
12	RH12	-6.82	-3.75	63.17	-3.76
13	RH13	4.12	10.53	77.87	4.90
14	RH14	3.92	4.25	88.40	11.11
15	RH15	9.67	23.85	77.83	4.87
16	RH17	-32.38	-31.12	19.99	-29.23
17	RH19	-7.08	18.27	38.26	-18.46
18	WH1	3.97	13.52	80.67	6.55
19	WH2	-3.6	-3.6	81.62	7.11
20	WH3	100	100	88.40	11.11
21	WH4	4.86	14.14	82.71	7.75
22	WH5	-17.49	-3.00	35.22	-20.25
23	WH6	3.98	16.51	76.88	4.31
24	WH7	7.39	15.97	88.40	11.11
25	WH8	11.05	31.95	80.63	6.52
26	WH9	2.71	5.58	88.40	11.11
27	WH10	-5.34	-3.18	74.44	2.88
28	WH11	-3.15	1.40	74.61	2.98
29	YH1	2.88	13.64	71.15	0.93
30	YH2	-25.73	-23.73	14.84	-32.26
31	YH3	-2.40	7.11	61.31	-4.86
32	YH4	-24.21	-22.16	17.21	-30.87
33	YH5	-1.06	4.14	41.90	-16.31
34	YH6	13.56	27.82	92.50	13.52
	Over all Mean	-5.69	3.48	60.07	-5.60

MP, Mid parent; BP, Better parent; Best P, Best parent.

TABLE 4: Stability analysis for total loss of bulbs in 4 M of storage.

Sl. No.	Hybrid/ Variety	KB mean per se	Rank	WB mean per se	Rank	SB mean per se	Rank	Overall mean per se	Rank	bi	S^2d
1	RH1	79.23		55.80		93.34		76.13		2.46	–0.0023
2	RH2	68.16		60.42		92.28		73.62		1.90	0.0009
3	RH3	88.39		67.35		87.59		81.11		1.58	–0.0012
4	RH4	61.53	III	64.33		68.02		64.63		0.15	–0.0015
5	RH5	66.06		50.27	II	38.94	I	51.76	II	–0.57	0.0228
6	RH6	91.67		89.14		100.00		93.60		0.45	–0.0022
7	RH8	44.21	I	44.38	I	59.49	III	49.36	I	1.10	0.0026
8	RH9	95.95		67.01		100.00		87.65		1.89	–0.0018
9	RH10	82.15		62.74		83.57		76.15		1.60	–0.0014
10	RH11	92.47		80.26		95.13		89.29		0.79	–0.0025
11	RH12	86.61		60.46		100.00		82.36		2.32	–0.0024
12	RH13	94.41		60.87		93.04		82.77		2.07	–0.0002
13	RH14	100.00		61.21		95.53		85.58		2.21	0.0012
14	RH17	63.69		66.86		49.79	II	60.12	III	–1.24	–0.0021
15	WH1	95.90		79.76		92.16		89.27		0.75	–0.0017
16	WH3	100.00		100.00		100.00		100.00		0.00	–0.0026
17	WH4	96.98		58.38		100.00		85.12		2.58	–0.0005
18	WH5	71.77		53.16	III	87.43		70.79		2.29	–0.0021
19	WH7	100.00		72.79		96.03		89.61		1.39	–0.0008
20	WH8	95.87		62.55		100.00		86.14		2.24	–0.0014
21	YH1	90.84		80.46		92.57		87.96		0.69	–0.0025
22	YH2	60.96	II	63.98		91.40		72.11		1.33	0.0078
23	YH3	85.62		89.62		100.00		91.75		0.34	–0.0007
24	YH4	62.21		96.67		89.01		82.63		–0.91	0.0185
25	YH5	75.32		79.38		87.10		80.60		0.30	–0.0006
26	16/26 GR3	89.75		96.54		78.58		88.29		–0.90	–0.0018
27	16/20	77.16		90.55		92.66		86.79		¯0.09	0.0014
28	16/21 GR3	96.35		77.74		94.65		89.58		1.07	–0.0017
	Total	2313.27		1992.66		2458.33		2254.75		27.82	0.0214
	Mean	82.62		71.17		87.80		80.53		0.99	0.0008

KB, *Kharif* Bengaluru; WB, *Winter* Bengaluru; SB, *Summer* Bengaluru.

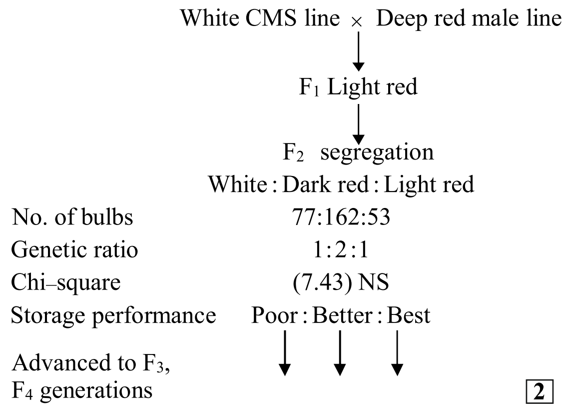
followed by RH5 (51.76%) and RH17 (60.12%) which assured in at least 3 or 2 cases of ranking respectively (Table 4). However, it was only RH8 which was found stable in its performance with unit regression coefficient (bi=1.10) with standard deviation (s^2d = 0.0026) nearing zero.

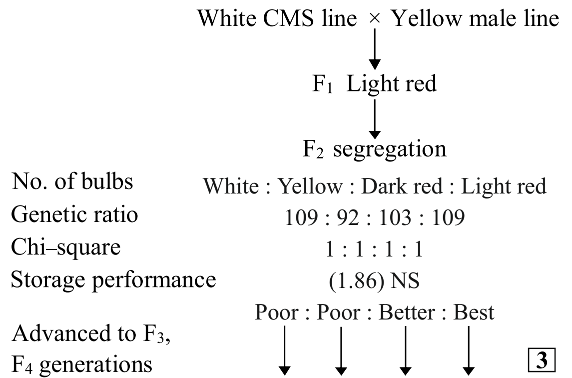
The DHs stored better (17.9% & 60.36% loss) than OP (27.02 % & 83.82% loss) in both 1 M and 4 M storage (Table 5).

TABLE 5: Performance of double haploids (DH) in 1 and 4 M of storage loss (%).

Sl. No.		*Kharif* 2020	
		1 M	4 M
Double haploid			
1	DH 16/25-8	19.70	57.59
2	DH(AB)16/25-2	16.10	63.14
	Mean	17.90	60.36
Check			
1	Virat	22.24	90.50
2	Marshal	31.80	77.14
	Mean	27.02	83.82

It was suggested by Madalageri (1983) that individual bulb selection in segregating population can be practised in developing good storage type onions. In that case, 2 hybrid populations were advanced to F_2 generation (Figs 2, 3). There were 3 phenotypes (white, dark red and light red) in white × red and 4 phenotypes (white, yellow, dark red and light red) in white × yellow cross. The bulbs of each phenotype were stored and selected the storer types and advanced





Figs 2 & 3: *Allium cepa.* 2. Pattern of inheritance in F_1 hybrid and its F_2 progenies in white and red crosses. 3. Pattern of inheritance in F_1 hybrid and its F_2 progenies in white and yellow crosses.

to F_3 and F_4 generations. It was generally found that light red bulbs of the segregating population stored better than other phenotypes. The selection process will continue up to F_6 or F_7 generation to establish a good storage type onion.

DISCUSSION

European and North American countries store their onions in cold storage (0–4°C) with 65 –70% RH throughout the year with least spoilage.

Tropical countries do not have cold chain facilities and experience lot of post-harvest losses (Tripathi & Lawande 2006). Probably, there is no variety/hybrid in the world which can stand ambient storage without loss in extended period of storage. Research efforts are going on in all the centers to breed the one with minimum losses in storage. Our results indicated that, yellow genotypes are worst affected than red and white ones. Similar opinion was also expressed by Bajaj et al. (1979) and Mondal & Pramanik (1992). In India, yellow onions are not much in cultivation. Red onions are produced for fresh consumption and white ones are used mostly for processing.

The genetic make up of onion genotypes towards storage losses is high (% heritability) implying less possibilities of selecting good storage type variety but genetic advance is low encouraging the breeder a corner of hope for selection (Madalageri 1983).

Widening the genetic make up of a genotype is one possibility towards achieving the goal. The random crossing of selected genotypes with good storage ability (developing PCH or selective breeding by F_1 development) did not make any significant difference in the present study although F_1 hybrids were better in storage than OP varieties or PCH.

In a highly cross pollinated crop like onion and also morphological structure of the flower which produces only 6 seeds/flower, it is not economical to produce F_1 seeds by hand pollination unlike in other vegetable crops. Therefore, CMS lines are involved to produce hybrids by using selective male parents and are popular in trade for higher yield and uniformity. However, studies on storage performance are very rare. In our study on heterosis breeding with 34 hybrids identified the best red hybrid, RH8 with negative heterosis in all the 4 components followed by RH12, RH17, WH5,YH2 and YH4 (on 3 components) in 1 M of storage. However, the extended period of storage for 4 M on crops grown in *kharif*, *winter* and *summer* seasons revealed that, the mean least per se losses were in RH8 followed by RH5 and RH17. The unit regression coefficient (bi) that indicates the stability of a genotype is only the parameter that can be relied upon to judge superiority of a hybrid for a good storage type and that was found in hybrid, RH8. This hybrid can be commercia-lized if other characters like high yield and uniformity of the bulbs are assured.

Breeding to bring in homozygocity (DH) in the genotype did help to some extent but not a fool proof to avoid storage loss. When a character is controlled by additive and dominant genes as shown in Table 3 and as revealed by Madalageri (1983) and Netrapal & Singh (1999), recurrent selection and/or single bulb selection in segregating population is more effective. Therefore, segregating populations indicated the possibilities of selecting the bulbs of best storers in F_2 generation and advanced them to further F_3 and F_4 generations. Futuring to further seed

enhancing up to F_6 or F_7 generation might fulfill the hope (Figs 2, 3).

Concluding the above effort, no single method of onion breeding will fully augment the post-harvest losses in onion. Long range efforts are needed to combine all possible methods of breeding to develop a variety/hybrid that can reduce the loss considerably if not 100%.

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