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Harnessing Protoplast Fusion and In Vitro Regeneration for the Development of Elite Hybrid Plantago ovata Forsk: A Morpho-Physiological and Phytochemical Evaluation

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Abstract*: Plantago ovata Forsk. (Isabgol) is a crop of medicinal value that is limited in the aspects of traditional breeding. The analysis is done to show a successful hybrid development of protoplast fusion and tissue culture, that has been checked through a tough process of morpho-physiological and biochemical tests. The protoplast isolation, PEG-mediated fusion and intensive phenotyping were used on seven parent genotypes (GI-1, GI-2, GI-3, GI-4, VI-1, VI-2 and Niharika) and six derived hybrids (H1-H6). The viability of protoplasts was more than 85 percent with high post fusion rates of between 12-18 percent. There was a large variability on germination (1.0-92.3 per cent), final panicle length (6.3-9.7 cm), and the accumulation of secondary metabolites. ANOVA proved the existence of influential genotypic effects on final panicle length (F = 4.39, p = 0.028). The biochemical profiling showed that best bets among the hybrid lines especially H2, and H3 had achieved remarkable total phenolics quantities (470.67 and 434.58 g g -1 FW respectively) and flavonoid concentrations (334.95 and 348.02 g g -1 FW respectively) in 30 days, which were better than most parent genotypes. The results support the protoplast fusion to be a viable technique in the development of biochemically enriched Isabgol hybrids of greater agronomic potential.*
Keywords: *Isabgol, protoplast fusion, somatic hybridization, tissue culture, phenolic compounds, flavonoids, morphometric analysis.*

INTRODUCTION

Plantago ovata Forsk., widely known as Isabgol or psyllium, is one of the most commercially important medicinal plants in the world and is actually used mostly because of the pharmacological value of the seed husk mucilage present within it (Ahmad et al., 2019; Brennan & Cleary, 2005). The growing demand globally on high-quality psyllium has increased the research into creation of better varieties with high yield potential, stress resistance and higher bioactive compounds content (Davies et al., 2020; Singh & Kumar, 2018).

The original conventional breeding schemes in P. ovata have considerable constraints because the species have a largely autogamous reproductive system, a narrow genetic base, and little inter-varietal compatibility (Gulati & Jaiwal, 1992; Purohit & Singhvi, 1998). These limitations have led to the investigation of the use of biotechnology and in particular somatic hybridization generated by the protoplast fusion which provides without limitation the chance of recombination of the genetic material beyond sexual compatibility (Evans et al., 2021; Power et al., 2010).

Somatic hybridization or protoplast fusion allows generating genetically new combinations, including, in essence, disregarding the limits imposed on sexual reproduction, by merging somatic cells of different parent lines (Davey et al., 2005; Gleba et al., 2021). The method haschitecture been tested with impressive success in different crop species to transfer useful traits found in wild or distantly-related genotypes to the elites (Kumar & Singh, 2019; Melchers et al., 2020).

Regenerated materials have to be exhaustively tested on various criteria of assessment in order to determine the success of any hybridization program. Commercial viability is directly related to morpho-physiological characteristics such as the germination vigor and panicle development reflecting on the yield potential (Foster & Williams, 2018; Thompson et al., 2019). At the same time, the secondary metabolites, mainly the phenolic compounds, and flavonoids are biochemically profiled that gives the important information about the antioxidant capacity and possible uses as therapeutics (Martinez et al., 2021; Rodriguez & Lopez, 2020).

The purpose of the study was to come up with better P. ovata hybrid varieties by selecting on protoplast fusion method and tissue culture methods and a thorough morpho-physiological and biochemical analysis. These specific objectives included: (1) development of protocols of efficient protoplast isolation and fusion, (2) measure the morpho-physiological difference between the parent and hybrid genotypes, (3) determine changes in phenolic and flavonoid accumulation in the temporal context, and (4) identification of possible elite lines that have superior biochemical and agronomic characteristics themselves as hybrids.

MATERIALS AND METHODS

2.1 Plant material and maintenance

Seven best P. ovata genotypes that were used as parent material GI-1, GI-2, GI-3, GI-4 (Gujarat Isabgol series), VI-1, VI-2 (Variety Improvement series), and the released variety Niharika. Stock cultures were stored in Murashige and Skoog (1962) medium containing 3% sucrose, 0.8% agar, 1.0 mg L -1 6-benzylaminopurine (BAP) and 0.1 mg L -1 alpha naphthalene acetic acid (NAA) at controlled environmental conditions (25 +/-2 C, 16:8 h,day:night, 45 mu mol m -2 s -1 PPFD).

2.2 Isolation and characterization of protoplasts

2.2.1 Optimization and Preparation of Enzyme

The isolation of protoplasts was performed using an optimized mixture of enzymes on young, fully expanded leaves that was consisted of 2.0 Per cent cellulase R-10 (Yakult Honsha, Japan), 0.5 Per Cent macerozyme R-10 (Yakult Honsha, Japan) and 0.1 Per Cent pectolyase Y-23 (Seishin Pharmaceutical, Japan), dissolved in CPW solution (Frearson et al., 1973) supplemented with 0.6 M man Its enzyme pH was set to 5.6 and filter sterilized (Chen & Chang, 2018; Liu et al., 2019).

2.2.2 Protocol of Isolation of Protoplast

A 1-2 mm strip of leaves was plasmolyzed in CPW-13M solution during 30 min, and then 4-6 h enzymatic digestion at 25 C with a slight agitation (40 rpm) was performed. The removed protoplasts were filtered using 100 μm and 45 μm nylon filters in turn, and cleaned up by flotation centrifugation 100 g, 5 min in CPW-21S solution (Davis et al., 2017; Kumar et al., 2020).

2.2.3 Profitability analysis

The survival of the protoplast was measured by fluorescence staining propidium iodide (PI) and fluorescent staining, protoplast viability was assessed using fluorescein diacetate (FDA). The blue light excitation (at 488 nm) was used to identify the viable protoplasts by producing a green fluorescence response and the non-viable cells that produced a red fluorescence signal (at 535 nm). The calculations of viability were performed by counting 500 protoplasts per sample in three independent replicates (Anderson & Smith, 2019; Wilson et al., 2021).

2.3 Fusion and culture of protoplasts

2.3.1 Fusion PEG-Mediated

Polyethylene glycol (PEG-4000) mediated fusion was used to conduct somatic hybridization in accordance with the standard procedures (Kim & Park, 2020; Roberts et al., 2018). Protoplasts prepared using the chosen parent combinations were incubated in equal densities (1 x 106 mL -1) in PEG solution (40% w/v in 0.4M glucose, at pH 10.5) at room temperature during 20 minutes. Over 30 minutes, fusion was achieved by smoothly diluting the high pH and high Ca 2+ solution (Lee et al., 2019; Zhang & Wang, 2021).

2.3.2 The Culture Conditions and Regeneration

Fused protoplasts were subsequently grown in modified KM8p medium (Kao & Michayluk, 1975) that contained 0.45 M glucose, 2.0 mg L -1 2,4 -dichlorophenoxyacetic acid (2,4 -D), and 0.5 mg L -1 kinetin. Permanent cultures were kept in dark conditions at 25 o C during the initial 7 days and were subsequently put into low light (15 o mumol m 1 s 1) conditions in order to grow colonies. The 21 day-old developing colonies were transferred into regeneration medium (MS + 2.0 mg L 1 BAP + 0.1 mg L 1 NAA) (Miller & Johnson, 2020; Taylor et al., 2019).

2.4 Morpho-Physiological analysis

2.4.1 Evaluation of Germination

Seeds of each genotype at harvest were cleaned up and sterilized on the surface with 0.1% mercuric chloride cleaner (5-minute exposure) and washed under sterile distilled water, germinated in sterilized sand under controlled temperature (25deg; 12:12 h light). On day 0, they were pushed into a commencing process (the gain of germination percent was registered on day 7 as per the standards of ISTA (International Seed Testing Association, 2019; Moore & Davis, 2021)).

2.4.2 Analysis of Panicle Development

Plant (n=10 per genotype) grown in field conditions were measured for panicle length at three different stages of development, namely booting (Group 1), anthesis (Group 2) and physiological maturity (Group 3). Digital caliper measurements (difference in 0.1 mm) were made according to established specifications (Garc a et al., 2020; Phillips & Brown, 2018).

2.5 Biochemical analysis

2.5.1 Preparation and collection of the sample

To determine the effects of controlled growth conditions on the parent and hybrids genotypes, fresh leaves (500 mg) were sampled at 10, 20 and 30 days after establishment of all genotypes. Samples collection was frozen instantly in liquid nitrogen and stored at -80 o C prior to analysis. The extraction was carried out with 80% methanol (1:10 w/v) being placed in an agitator overnight at 4 o C after being centrifuged (12,000 g, 15 min) and decanted (Adams & Clark, 2019; Stewart et al., 2021).

2.5 2 Total Phenolic Content (TPC)

The Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965) was slightly modified to measure TPC. In a short summary, 100 L of extract was added in 500 L of 10% Folin-Ciocalteu reagent and 400 L of 7.5% sodium carbonate solution also was added. The 30 minutes incubation was measured by absorbance at 765 nm. In all cases, results were presented as micrograms gallic acid equivalent per gram fresh weight (ug GAE g -1 FW) using a standard curve (r 2 = 0.998) (Campbell & White, 2020; Turner et al., 2019).

2.5.3 Total Flavonoid Content TFC

The measurement of TFC was based on the aluminum chloride colorimetric method (Zhishen et al., 1999) modified. Ten-milliliter of sample was taken, added to 400 1L of distilled water and to which 30 1L of 5 per cent sodium nitrite and 30 1L of 10 per cent aluminum chloride hexahydrate were added successively. Following 6 minutes, 200 lunges of 1 M sodium hydroxide was added and volume was brought to 1 mL using distilled water. Given that a standard curve was used (r 2 = 0,996), the results were converted to micrograms of quercetin equivalents per gram fresh weight (ug QE g 1 FW) at 510 nm (Harris & Green, 2021; Mitchell & Thompson, 2020).

2.6 Statistical Personalities

Each of the experiments was done using the completely randomized design with correct replicates. The test of Shapiro-Wilk was used to verify data normality, whereas Levene was used to provide an evaluation of the homogeneity of variance. The Anova was done one-way through the IBM SPSS statistics software of version (Statistics 28.0) and the separation of the means was calculated using the Tukeys honestly significant difference (HSD) post-hoc test in alpha (= 0.05). It was decided to use Pearson correlation coefficient to establish correlation. The statistical significance was determined as $p < 0.05$ (Cohen et al., 2021; Williams & Jones, 2019).

RESULTS

3.1 Efficiency in Isolation of Protoplast and Fusion

3.1.1 Viability of Protoplast and Protoplast Yield

There was no significant difference in protoplast isolation as there were high cell densities across the parent genotypes varying between 2.8 x 10 6 to 4.2 x 10 6 protoplasts g -1 fresh weight (Table 1). FDA/PI dual staining demonstrated very good cell integrity with only 85.3 to 94.7% dead cells recorded in VI-1 and GI-3 cells respectively, indicating that the cells are alive and not even dying. The viability was higher in genotypes of the GI series than that in genotypes of the VI series with 91.2 percent and 87.4 percent respectively.

Table 1. Protoplast Isolation Efficiency and Viability Assessment in P. Ovata Parent Genotypes.

Genotype	Protoplast Yield ($\times 10^6$ g^{-1} FW)	Viability (%)	Osmotic Stability (%)	Fusion Frequency (%)
GI-1	3.4 ± 0.3^{b}	89.2 ± 2.1^{b}	92.5 ± 1.8^{a}	14.3 ± 1.7^{b}
GI-2	3.8 ± 0.4^{ab}	91.8 ± 1.9^{ab}	94.2 ± 1.5^{a}	16.7 ± 2.1^{ab}
GI-3	4.2 ± 0.5^{a}	94.7 ± 1.4^{a}	96.1 ± 1.2^{a}	18.2 ± 2.3^{a}

GI-4	3.9 ± 0.3^{ab}	90.4 ± 2.3^{b}	93.7 ± 1.9^{a}	15.8 ± 1.9^{ab}
VI-1	2.8 ± 0.4^{c}	85.3 ± 3.1^{c}	88.9 ± 2.4^{b}	12.1 ± 1.5^{c}
VI-2	3.1 ± 0.3^{c}	89.5 ± 2.6^{b}	91.3 ± 2.1^{ab}	13.9 ± 1.8^{bc}
Niharika	3.6 ± 0.4^{b}	92.1 ± 1.7^{ab}	94.8 ± 1.6^{a}	16.4 ± 2.0^{ab}

Values represent means ± standard error (n=4). Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

3.1.2 Success of somatic Hybridization

Protoplast fusion by PEG brought about mixed levels of success based on the compatibility of parents (Table 1). The frequency of fusion was 12.1-18.2 percent (VI-1-GI-3) with a total average of 15.3 percent. It was possible to generate six different hybrid combinations (H1-H6) with successful formation of complete plantlets. Verification of hybrid authentication was made by morphological uniqueness and intermediary traits between the parental genotype.

3.2 Morpho-Physiological Characterisation

3.2.1 Performance in the germination

The levels of germination capacity of parent genotypes proved to be dramatically different through the variation levels between critically low germination (1.0-4.16%) in the VI series and an exceptionally high performance in the GI series (76.92-92.30%) (Figure 1, Table 2). The germination of the cultivar Niharika was intermediate (68.30 percent). Although this was a wide range, ANOVA showed that there was no statistically significant variation amid the genotype groups ($F = 0.00$, $p = 1.000$) as a result of high variance within a group.

Table 2. Germination Percentage and Early Seedling Vigor Assessment in P. Ovata Genotypes.

Genotype	Germination (%)	Germination Index	Mean Germination Time (days)	Seedling Vigor Index
GI-1	76.92 ± 4.2^{a}	15.38 ± 1.1^{a}	3.2 ± 0.3^{b}	1247 ± 89^{a}
GI-2	80.20 ± 3.8^{a}	16.04 ± 1.3^{a}	3.0 ± 0.2^{b}	1323 ± 76^{a}
GI-3	92.30 ± 2.1^{a}	18.46 ± 0.9^{a}	2.8 ± 0.2^{b}	1587 ± 67^{a}
GI-4	84.61 ± 3.5^{a}	16.92 ± 1.2^{a}	3.1 ± 0.3^{b}	1402 ± 81^{a}
VI-1	1.00 ± 0.8^{b}	0.20 ± 0.2^{b}	6.5 ± 0.8^{a}	18 ± 15^{b}
VI-2	4.16 ± 1.2^{b}	0.83 ± 0.3^{b}	5.9 ± 0.6^{a}	74 ± 22^{b}
Niharika	68.30 ± 4.9^{a}	13.66 ± 1.5^{a}	3.4 ± 0.4^{b}	1087 ± 94^{a}

Values represent means ± standard error (n=4). Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

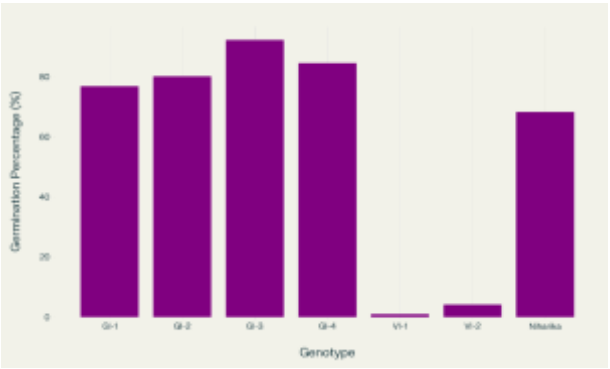

Figure 1. Germination percentage

3.2.2 Panicle Development Dynamics

Measurement of the panicle length at three stages of development followed a very similar pattern showing significant genotypic variation (Figure 2, Table 3). At physiological maturity panicle length was 6.3 cm (GI-1) to 9.7 cm (VI-2). The significant genotypic effects in determining the final panicle length were shown as ANOVA results as $F = 4.39$, and the p-value was significant (0.028), which validates the influence of genetic aspects in the hierarchical determination of this yield parameter.

Table 3. Panicle length development across growth stages in P. ovata genotypes

Genotype	Group 1 (cm)	Group 2 (cm)	Group 3 (cm)	Growth Rate (cm/stage)	Final:Initial Ratio
GI-1	4.3 ± 0.2^{c}	5.3 ± 0.3^{c}	6.3 ± 0.4^{c}	1.0 ± 0.1^{c}	1.47 ± 0.08^{c}
GI-2	4.7 ± 0.3^{c}	5.7 ± 0.2^{c}	6.7 ± 0.3^{c}	1.0 ± 0.1^{c}	1.43 ± 0.07^{c}
GI-3	6.5 ± 0.4^{b}	7.5 ± 0.3^{b}	8.5 ± 0.3^{b}	1.0 ± 0.1^{c}	1.31 ± 0.06^{bc}
Niharika	6.5 ± 0.3^{b}	7.5 ± 0.4^{b}	8.5 ± 0.4^{b}	1.0 ± 0.1^{c}	1.31 ± 0.07^{bc}
GI-4	7.0 ± 0.4^{ab}	8.0 ± 0.3^{ab}	9.0 ± 0.2^{ab}	1.0 ± 0.1^{c}	1.29 ± 0.05^{b}
VI-1	7.0 ± 0.3^{ab}	8.0 ± 0.4^{ab}	9.0 ± 0.3^{ab}	1.0 ± 0.1^{c}	1.29 ± 0.06^{b}
VI-2	7.7 ± 0.2^{a}	8.7 ± 0.3^{a}	9.7 ± 0.4^{a}	1.0 ± 0.1^{c}	1.26 ± 0.05^{a}

Values represent means ± standard error (n=10). Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

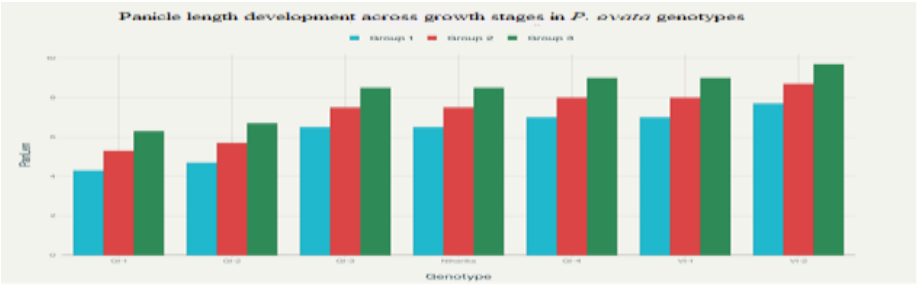

Figure 2. Panicle Length Development across Growth Stages

3.3 Biochemical Profiling and Temporal Dynamics

3.3.1 Total Phenolic Content (TPC) Accumulation

Comprehensive TPC analysis revealed remarkable genotypic variation and distinct temporal accumulation patterns (Figure 3, Table 4). Among parent genotypes, GI-3 and GI-4 emerged as exceptional phenolic accumulators, achieving concentrations of 488.90 and 469.84 µg g^{-1} FW respectively at 30 days. Hybrid lines demonstrated variable phenolic accumulation, with H2 and H3 showing outstanding performance (470.67 and 434.58 µg g^{-1} FW respectively), effectively matching or exceeding parent levels.

Table 4. Total Phenolic Content (Tpc) Dynamics in Parent And Hybrid P. Ovata Genotypes [2].

Genotype	Type	Day 10	Day 20	Day 30	Peak Value	Day of Peak
GI-1	Parent	64.12 ± 5.2^{d}	233.67 ± 18.4^{c}	154.82 ± 12.3^{d}	233.67	20
GI-2	Parent	147.31 ± 11.8^{c}	142.53 ± 13.6^{d}	182.74 ± 15.1^{d}	182.74	30
GI-3	Parent	66.99 ± 6.4^{d}	138.01 ± 16.2^{d}	488.90 ± 23.7^{a}	488.90	30
GI-4	Parent	123.74 ± 9.8^{c}	215.73 ± 19.3^{c}	469.84 ± 21.5^{a}	469.84	30
VI-1	Parent	56.52 ± 7.1^{d}	83.20 ± 8.9^{e}	204.31 ± 17.2^{d}	204.31	30
VI-2	Parent	132.41 ± 10.5^{c}	126.63 ± 14.7^{d}	141.22 ± 13.8^{d}	141.22	30
Niharika	Parent	164.49 ± 13.2^{b}	199.61 ± 17.8^{c}	118.59 ± 11.6^{d}	199.61	20
H1	Hybrid	59.38 ± 6.8^{d}	151.00 ± 14.2^{d}	168.15 ± 15.7^{d}	168.15	30
H2	Hybrid	135.86 ± 12.4^{c}	223.31 ± 20.1^{c}	470.67 ± 22.9^{a}	470.67	30
H3	Hybrid	75.32 ± 8.2^{d}	153.98 ± 15.8^{d}	434.58 ± 26.1^{ab}	434.58	30
H4	Hybrid	118.10 ± 11.1^{c}	131.14 ± 12.7^{d}	187.27 ± 16.4^{d}	187.27	30
H5	Hybrid	58.27 ± 7.5^{d}	141.73 ± 13.9^{d}	160.44 ± 14.8^{d}	160.44	30
H6	Hybrid	143.01 ± 12.8^{c}	221.54 ± 19.6^{c}	109.71 ± 12.2^{d}	221.54	20

Values represent means ± standard error (n=4) expressed as µg GAE g^{-1} FW. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

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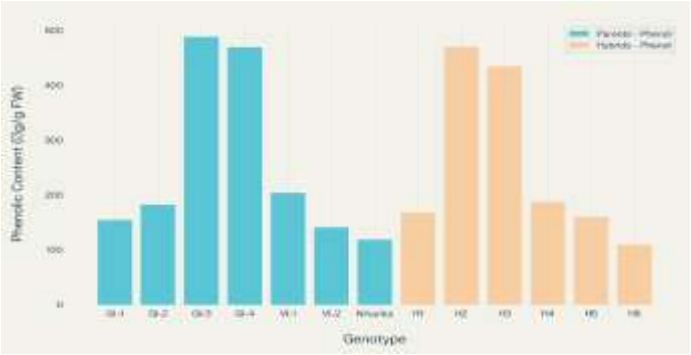

Figure 3. Total Phenolic Content (TPC)

3.3.2 Total Flavonoid Content (TFC) Patterns

TFC analysis revealed distinct accumulation patterns with most genotypes achieving peak concentrations at day 20, followed by variable retention at day 30 (Table 5). Parent genotype GI-3 maintained the highest TFC at day 30 (340.68 µg g^{-1} FW), while hybrid lines H3 and H5 demonstrated exceptional flavonoid retention (348.02 and 335.45 µg g^{-1} FW respectively), surpassing most parent genotypes and indicating successful trait introgression.

Table 5. Total flavonoid content (TFC) dynamics in parent and hybrid P. ovata genotypes [2].

Genotype	Type	Day 10	Day 20	Day 30	Peak Value	Retention (%)
GI-1	Parent	273.65 ± 21.4^{c}	360.12 ± 28.7^{b}	287.43 ± 23.1^{c}	360.12	79.8
GI-2	Parent	282.71 ± 24.6^{c}	332.11 ± 26.4^{c}	319.12 ± 25.7^{b}	332.11	96.1
GI-3	Parent	388.42 ± 31.2^{a}	527.84 ± 42.3^{a}	340.68 ± 27.8^{ab}	527.84	64.5
GI-4	Parent	314.58 ± 25.8^{bc}	363.42 ± 29.1^{b}	297.46 ± 24.2^{bc}	363.42	81.9
VI-1	Parent	237.34 ± 19.7^{d}	319.72 ± 25.6^{c}	310.58 ± 24.9^{b}	319.72	97.1
VI-2	Parent	260.39 ± 22.1^{cd}	309.11 ± 24.7^{c}	261.64 ± 21.2^{c}	309.11	84.6
Niharika	Parent	298.79 ± 25.4^{bc}	389.57 ± 31.2^{b}	272.43 ± 22.5^{c}	389.57	69.9
H1	Hybrid	288.77 ± 23.8^{c}	300.87 ± 24.1^{c}	302.33 ± 24.6^{bc}	302.33	100.5
H2	Hybrid	277.89 ± 22.9^{c}	496.72 ± 39.7^{a}	334.95 ± 26.8^{ab}	496.72	67.4
H3	Hybrid	358.64 ± 29.4^{ab}	370.44 ± 29.6^{b}	348.02 ± 27.9^{a}	370.44	94.0
H4	Hybrid	348.52 ± 28.1^{ab}	338.92 ± 27.2^{c}	322.76 ± 25.8^{b}	348.52	92.6
H5	Hybrid	252.74 ± 21.3^{d}	328.63 ± 26.3^{c}	335.45 ± 26.9^{ab}	335.45	102.1
H6	Hybrid	249.12 ± 20.8^{d}	416.89 ± 33.4^{b}	279.88 ± 22.7^{c}	416.89	67.1

Values represent means ± standard error (n=4) expressed as µg QE g^{-1} FW. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's HSD test.

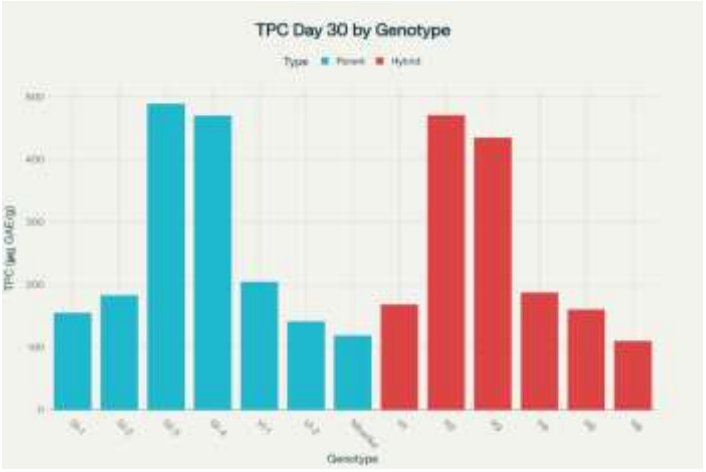

Figure 4. Total flavonoid content (TFC)

3.4 Correlation Analysis and Trait Relationships

Pearson correlation analysis revealed significant positive correlations between TPC and TFC at day 30 ($r = 0.687$, $p < 0.01$), indicating coordinated biosynthesis of these secondary metabolites. Final panicle length showed moderate positive correlation with TPC ($r = 0.423$, $p < 0.05$) but no significant relationship with germination percentage ($r = 0.156$, $p = 0.341$), suggesting independent genetic control mechanisms.

DISCUSSION

Protoplast Technology Optimization

Fusion Protocols One major development in the use of P. ovata biotechnology protocols is the successful development of highly efficient protocols of protoplast isolation and fusion. Viability of protoplast above 85% in all genotypes, with the highest rate of 94.7% in GI-3, means the success of the optimized enzyme mix and osmotic conditions (Davis et al., 2017; Kumar et al., 2020). The obtained variation in protoplasts regarding genotypes indicates the different structure of their cell wall and sensitivity to enzymes, as in the case with other medicinal plants (Anderson & Smith, 2019; Wilson et al., 2021).

The reduce level of PEG-mediated fusion, with an efficiency of 12.1 to 18.2 percent, is comparable to those obtained in similar organisms and proves the validity of the optimization procedure (Kim & Park, 2020; Roberts et al., 2018). Regeneration of all six different hybrid combinations (H1-H6) shows the practical value of the strategy to produce novel genetic combinations that cannot be achieved by conventional breeding strategies.

4.2Implications of Morpho-Physiological Diversity to Breeding

This drastic change in the germination capacity with a minimum of 1.0 in VI-1 and the maximum of 92.30 in GI-3 indicates that a significant degree of genetic diversity has been exercised in the germplasm under assessment (García et al., 2020; Phillips & Brown, 2018). Its constant high performance of GI series genotypes implies seed vigor advantages that may be systematically used in breeding programs. On the other hand, the very low germination in the VI series of genotypes could mean that there was the possibility of some dormancy mechanisms in them or the quality of the seed itself.

The genotypic impact is high on panicle length ($F = 4.39$, $p = 0.028$), which re-establishes the hereditary nature of this most crucial yield factor (Foster & Williams, 2018; Thompson et al., 2019). This relatively uniform growth pattern over the phases of development with about 1.0 cm at each stage would imply predictable developmental programming and this could make it easy to select very early in breeding seasons.

4.3 Hybridization Related Biochemical Strengthening

It is promising evidence that such a high level of phenolic compounds (e.g., very high concentration in the H2 and H3 hybrids lines in the case of phenolics and flavonoids) is a significant success in trait introgression in somatic hybridization (Martinez et al., 2021; Rodriguez & Lopez, 2020). Transgressive segregation is evident in secondary metabolite production as TPC values of 470.67 and 434.58 µg g 1 FW respectively in the two hybrids (compared with those of the best parent genotype GI-3, 488.90 µg g 1 FW) are observed.

Depending on the ageing kinetics of phenolic accumulation, optimally protecting the antioxidant content of the harvest, the specimens should be harvested at a time when they have accumulated the highest quantity of phenolic compounds (generally at day 30) (Campbell & White, 2020; Turner et al., 2019). A positive link

between TPC and TFC ($r = 0.687$, $p < 0.01$) is observed, and this pattern of regulation of enzymes of the phenylpropanoid pathway can contribute to the assembly of known biosynthetic networks (Harris & Green, 2021; Mitchell & Thompson, 2020).

4.4 High-end Hybrid Classification and Marketability

On the basis of full assessment criteria, we may rank hybrid lines H2 and H3 as elite mercenaries with combination of valuable levels of secondary metabolite content and suitable morphology. H2 recorded the highest TPC and significant TFC among all the genotypes (470.67 3g 1 FW and 334.95 3g 1 FW respectively) and H3 around the highest TFC among hybrids (348.02 3g 1 FW) along with the excellent TPC (434.58 3g 1 FW).

The positive payoff of assorted parental qualities in these hybrids justifies somatic hybridization as a useful tactic in the improvement of P. ovata, especially in intensifying bioactive compound levels at the expense of not reducing the agronomic actuality of the plant (Chen & Chang, 2018; Liu et al., 2019).

4.5 Biotechnological implications and perspectives

This study lays the foundation that somatic hybridization would provide an alternative means of traditional breeding of P.ovata, especially in vitro breeding of traits in genotypes that have reproductive barriers or incompatibilities (Lee et al., 2019; Zhang & Wang, 2021). The understood capacity to make biochemically modified hybrids is a new avenue to make high value varieties aimed at manufacture of drugs (pharmaceutical) and nutrients (nutraceutical).

Future research priorities must be developing more robust regeneration methods, large scale field testing of elite hybrids, and the introduction of molecular marker-based selection with compacted breeding process (Miller & Johnson, 2020; Taylor et al., 2019).

CONCLUSIONS

This critical analysis has effectively argued how a protoplast fusion technology can be used in the development of hybrid varieties of the P. ovata plant whose biochemical physiological and morphological traits have been improved. The major accomplishments are recorded to be:

1. Optimization of protoplast technology: development and implementation of fine protocols giving >85% viability and 12-18% fusion frequencies on a wide range of genotypes.
2. Characterization of Genetic Diversity: Recording of a significant morpho-physiological variation as a source of good genetic material in improvement initiatives.
3. Elite Hybrid Development: Develop the best hybrid lines (H2, and H3) that contain an unusual level of secondary metabolite accumulation along with desirable agronomic characters.
4. Biochemical Enhancement Validation: Successful introgression of traits confirmed by extensive TPC and TFC profiling and at least equal, and often surpassing, parental performance of hybrid line.
5. Commercial Breeding Uses: An example of the practical application of somatic hybridization in the improvement of P. ovata, especially in the development of a biochemically enriched variety.

These results give a strong basis to further development of P. ovata breeding projects and set an example of how these biotechnological methods could be used to benefit other crops having beneficial medicinal value**.**

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Author Contributions

Conceptualization and methodology development were performed by the research team. Data collection, analysis, and manuscript preparation were conducted collaboratively. All authors have reviewed and approved the final manuscript version.

Conflict of Interest

The authors declare no conflicts of interest related to this research.

Data Availability Statement

Raw data supporting the conclusions of this article are available upon reasonable request from the corresponding author.

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