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ORIGINAL ARTICLE

Exogenous polyamines improved chloroplast count and indirect organogenesis of Indian pea (*Pisum sativum* L.) cv. Ageta 6

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Abstract An effective callus-dependent indirect organogenesis protocol was standardized in recalcitrant Indian pea cv. Ageta 6 using cotyledonary node explant. This research highlighted the positive action of different polyamines (PA) such as spermidine (SPD), spermine (SPM), and putrescine (PUT) along with different plant growth regulators (PGRs) such as 2, 4-dichlorophenoxyacetic acid (2, 4-D), 6-benzyladenine (BA), and 1-naphthaleneacetic acid (NAA) on the enhancement of peas indirect organogenesis with callus induction, multiple shooting, and rooting parameters. The callus induction experiment demonstrated, 30 mg/L of PUT and 3 mg/L of 2, 4-D supplemented callus induction media has produced the highest green and white, friable pea callus induction with the highest explant response (46.26%). In the course of shoot multiplication study from the callus pieces of pea revealed that the highest multiple shooting efficiency (12.66 shoots/callus piece) under 20 mg/L of SPD and 1.5 mg/L BA treatments along with the highest explant response (53.66%) and shoot length (3.93 cm /shoots). The highest shoot elongation (6.20 cm length/shoots) was achieved under 1 mg/L GA_3 enriched shoot elongation media with maximum explant response (70%) even without polyamine assistance. The improved rooting analysis demonstrated, the highest root induction (10.86 roots/shoots) under 25 mg/L of PUT along with 0.6 mg/L of NAA supplemented rooting media with maximum root length (3.90 cm/shoots) and explant response (40%). Rooted plantlets were hardened effectively with a survival rate of 92%. The maximum callus induction (46.26%), shoot multiplication (12.66 shoot/callus piece), and root induction (10.86% root/shoot) were recorded in pea plant by the application of exogenous polyamines, which was approximately two to three-fold higher than the regular PGRs assisted indirect pea regeneration. The RAPD and SCoT molecular marker analysis justified the somoclonal variations free genomic steadiness in regenerated plants. Simultaneously, the polyamines-assisted regenerated pea plants showed three-fold improved photosynthetic (chlorophyll a, b and carotenoid) and two-fold improved antioxidant (DPPH, H_2O_2, and NO assay) profiles. Laser scanning confocal microscopy captured that the PA-assisted pea regenerants had a tremendous upsurge in the digit of precise chloroplasts compared to the control plant.

Keywords Antioxidant · Chlorophyll content · Chloroplast count · Cotyledonary node · Genetic fidelity · Indirect organogenesis · Pea · Polyamines

Abbreviations

PA	Polyamine
PGRs	Plant growth regulators
2, 4-D	2, 4-dichlorophenoxyacetic acid
BA	6-benzyladenine (or) benzyladenine
NAA	1-naphthaleneacetic acid
cv.	Cultivar
RAPD	Random amplified polymorphic DNA
SCoT	Start codon targeted polymorphism
SPD	Spermidine
SPM	Spermine
PUT	Putrescine

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Introduction

Pea (*Pisum sativum* L.), commonly known as field pea or garden pea or green pea, is an herbaceous annual crop plant belonging to the family Fabaceae. Pea has been the second-largest leguminous plant cultivating for centuries as a significant crop for fodder and human consumption (Cousin, 1997). It is known as "poor man's meat" since its vast protein and high carbohydrates, vitamins, and antioxidants. Asia has been meeting three-quarters (86.4%) of the world's pea demand (1.6 crore tonnes) by continent in pea production for the past decade (2010–2020). In terms of countries, China ranks first (1.0 crore tonnes), India second (45.1 lakh tonnes) and the United States of America third (3.0 lakh tonnes) globally (FAOSTAT 2010–20). Although the area under pea cultivation is increasing worldwide, biotic (bacteria, virus, fungus, nematodes, and insects) and abiotic (drought, heavy metals, pH, temperature, salinity, and pesticides) stresses are the significant barriers to increasing the global pea production (Grunwald et al., 2004). The substantial role of pea cultivation is to fulfill the nutritional demand of the population with increased resistance against biotic and abiotic stresses without compromising yield parameters (Ochatt et al., 2000a; International Year of Pulses-2016); Genetic modification technology paves the way to achieve this goal (Christou, 1997; Ochatt et al., 2000b). Although every successful plant genetic modification technology requires a stable regeneration system, the pea's in vitro regeneration performance is poor, due to its stable genetic configuration. Even though some of the most important plant tissue culture (PTC) and PTC dependent genetic transformation researches has been documented, such as Puonti-Kaerlas et al., (1990), Schroeder et al., (1993), Ochatt et al., (2000a, b), and Svabova et al., (2005, 2008), there is still a need to upgrade pea output by figuring out a better way to cultivate peas to balance global pea demand. Therefore, standardizing a reliable and successful regeneration protocol is a prerequisite for improving pea genetic alignment (Ozcan et al., 1993; Christou, 1997; Ochatt et al., 2010). Pea tissue culture provided exciting opportunities to improve pea breeding than conventional approaches (Smykal, 2014). Comparatively, indirect organogenesis has attracted much attention in pea regeneration due to the stable, elevated dynamic organogenesis and clonal efficiency. Polyamines have a critical function in promoting plant morphogenesis due to their distinct low molecular weight, aliphatic nitrogenous, and polycationic characteristics. It is the essential that the natural chemical compound actively involved in rejuvenating plants' cellular functions, such as protein synthesis, DNA replication, phytoimmunity, and rapid cell division processes. It has been suggested by many researchers that plant growth doubles when polyamines are given exogenously to plants along with plant growth hormones (Kakkar & Sawhney, 2002; Kuehn & Phillips, 2005). So far, only a few studies have commendably established the callus-dependent indirect organogenesis pathway in pea.

Moreover, Polyamines protect and enhance the number of healthy chloroplasts and the stabilization of photosynthetic apparatuses (PSII), such as thylakoid membranes and light-harvesting complexes (LHC), resulting in improved total chlorophyll content in plants (Ajithan et al., 2019; Baryla et al., 2001; Galston et al., 1997; Kakkar & Nagar, 1996; Kaur-sawhney & Galston, 1979; Lee et al., 1997; Shu et al., 2012; Zhang et al., 2009). With the application of exogenous polyamines, we discovered better callus independent direct organogenesis (shoots and roots) from the cotyledonary node explant of Indian pea cultivar Ageta 6, with improved chloroplast count, total chlorophyll, and antioxidant profile (Ajithan et al., 2019). As a result of the data mentioned above, the first time we investigated the indirect organogenesis of Indian pea cv. Ageta 6 with the stimulation of different exogenous polyamines (SPD, SPM, and PUT) and PGR (2, 4-D, BA, and NAA) in this study. The genetic fidelity investigation was performed utilizing RAPD and SCoT molecular markers to investigate the genetic inimitability of the regenerated pea plant. Aside from that, the chloroplast number, the profile of chlorophyll, and antioxidants of regenerated pea plants induced by polyamines were examined microscopically and biochemically.

Materials and methods

Plant materials and explant preparation

Popular Indian pea cv. Ageta 6 was purchased from the National Seed Corporation (NSC), Ooty, Tamil Nadu, India, and used in this indirect organogenesis experiment. The seeds were sterilized by using the chlorination method recommended by Di et al., (1996) and Ajithan et al., (2019) by disinfecting the seeds in a separate desiccator with 2 ml of concentrated hydrochloric acid (HCL) and 30 ml of sodium hypochlorite (NaOCl) induced chlorine gas for 3–4 h of incubation. The cotyledonary node explants (0.3×0.2 inches) were prepared from three-day-sterilized water-soaked pea seeds by dissecting the seed coat, shoot tip and root tip. All the growth media, including callus induction, multiple shooting, shoot elongation, and root induction, were made by full-strength MS (Murashige & Skoog, 1962) media with solidification agent 0.8% of Agar Agar and autoclaved under 110 kPa for 30 min at 121 °C. The chemicals which are utilized for this entire research were purchased from HiMedia®, Mumbai, India. As this indirect organogenesis of peas has been designed based on the direct organogenesis protocol we have already standardized (Ajithan et al., 2019) on Ageta 6; hence we utilized the same

plant growth regulators (PGRs) in this research that made a significant contribution in previous investigation on multiple shooting, shoot elongation, and root induction.

Effect of PGRs on callus induction, shoot multiplication, shoot elongation, and root induction

After preparation, the explant were inoculated in the callus induction medium (CIM), constituting various concentrations (1–6 mg/L) of 2, 4-D, and incubated for 3 weeks. After callus induction, the calluses were transferred to Shoot Multiplication Medium (SMM) constituted with various concentrations (0.5–3.0 mg/L) of BA for three weeks. After shootings, the multiplied shoots were sub-cultured on Shoot Elongation Medium (SEM) for 4 weeks, supplemented with various concentrations (0.2–1.2 mg/L) of GA_3. After shoot elongation, the elongated shoots were separated and shifted into Root Induction Medium (RIM) at various concentrations of NAA (0.2–1.2 mg/L) for 4 weeks. All experiments were accomplished under 16/8 h of cool fluorescent photoperiod with 50 µmol m^{-2} s^{-1} irradiances at 25 ± 2 °C.

Effect of polyamines on callus induction, shoot multiplication, and root induction

The polyamine stock solutions were made and sterilized using a filter sterilization procedure followed by Ajithan et al., (2019) using a 0.22 µm sterile syringe-driven filter (HiMedia®, Mumbai, India) and dissolved in the growth media at 45 °C in a sterile environment. The influence of polyamines on callus induction, shoot multiplication, and root induction in pea was studied with exposing the respective explant (explant/callus pieces/shoots) to various concentrations (5–40 mg/L) of three different polyamines, SPD, SPM, and PUT, along with the standardized concentration of respective PGRs (2, 4-D/BA/NAA). Except for the optimal dose of GA_3, no polyamines were added for the shoot elongation study. Polyamine-free PGR assisted regeneration was continued as a simultaneous control experiment in each analysis.

Hardening and acclimatization

After the rooting experiments, the in vitro regenerated plantlets were removed from the culture tubes, cleaned thoroughly with sterile distilled water, and transferred to paper cups filled with the mix of soil, sand, and soil rite (1:1:1 v/v/v ratio). All the plants were maintained under 80% moisture condition in a growth chamber for two weeks. To maintain humidity, the plantlets were wrapped with polythene bags, and when they showed signs of acclimatization, the plastic covers were removed and the plants were transferred to the greenhouse.

Statistical analysis

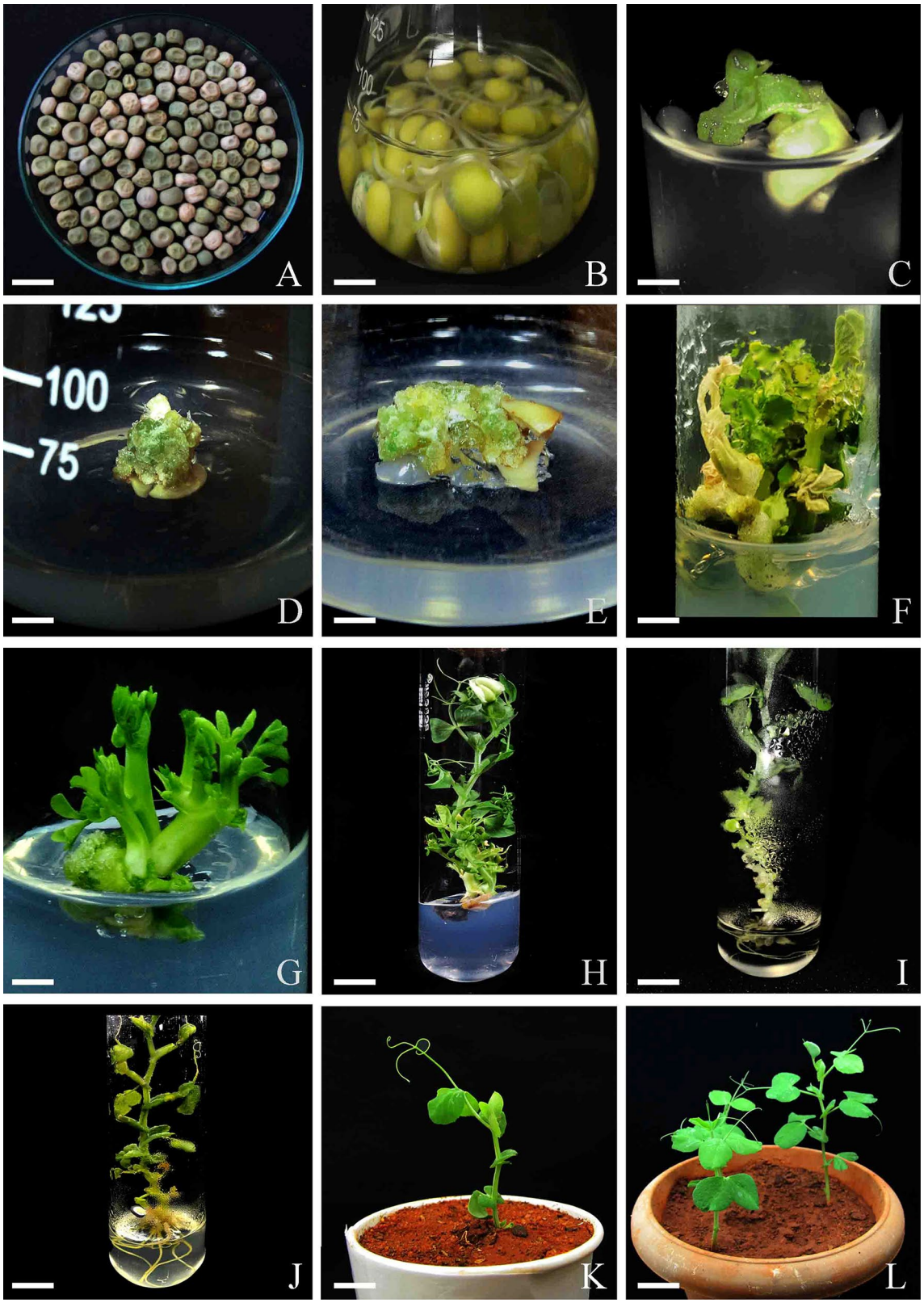
Applying 100 explant per treatment, each experiment was repeated three times. The data were analyzed via the Duncan Multiple Range Test and one-way ANOVA (DMRT). SPSS 20 (SPSS Inc, Armonk, New York, USA) was used for statistical analysis (with a *P* value less than 0.05), and graphs were made with Origin (OriginPro 8, MicroCal Inc, Westborough, Massachusetts, USA) on Operating System Windows 8.0.

Genetic stability analysis by RAPD and SCoT markers

The molecular markers analysis of Random amplified polymorphic DNA (RAPD) and Start Codon Targeted (SCoT) polymorphism were employed to ensure the genetic originality of the regenerated pea. Genomic DNA was isolated from nine randomly selected PA-assisted regenerated plants and one mother plant by using a DNA isolation kit (Sigma Aldrich, St. Louis, USA). The 9 RAPD specific primers were applied for the RAPD analysis, while the 17 SCoT specific primers were used for the SCoT amplification. Under the thermocycler PTC-100TM (MJ Research Inc., Waltham, USA), the PCR reaction was set for RAPD analysis as initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min. The SCoT specific PCR was applied using the same RAPD PCR scheme with only one variation in annealing at 50 °C for 1 min. The amplified PCR products were analyzed with agarose (1.2%) gel electrophoresis, and the emitted bands were scored by the Agarwal et al., (2015) procedure.

Photosynthetic, chloroplast, and antioxidant analysis

The total chlorophyll (chlorophyll a, b and carotenoid) of polyamine assisted, and non-polyamine assisted (control) pea regenerants were examined by using Aremu et al., (2012) protocol. The microscopic arrangement for chloroplast count was studied by Baryla et al., (2001) protocol in Main Beam Splitter (MBS) with 488 nm filter equipped laser scanning confocal microscopy (Carl Zeiss Microscopy GMBH, Jena, Germany). Antioxidant profiles of polyamine assisted and non-polyamine assisted (control) regenerated plants were assessed by using hydrogen peroxide (H_2O_2), 1,1-diphenyl2-picryl-hydrazyl (DPPH), and nitric oxide (NO) scavenging activity assays, as suggested by Jayaprakasha et al., (2004); Shen et al., (2010) and Sonawane et al., (2010). Ascorbic acid was utilized as the standard in all of the antioxidant screening. H_2O_2, DPPH, and NO absorbance levels were measured at 546, 517, and 230 nm, respectively.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◂**Fig. 1** **A** The healthy pea seeds (cv. Ageta 6) (bar 1 cm); **B** The three days imbibed pea seeds under sterile water (bar 1 cm); **C** The inoculated cotyledonary node explant without shoot tip and root tip (bar 0.3 mm); **D** The callus emergence from the cotyledonary node explant of pea under 3 mg/L of 2, 4-D assisted callus induction media (bar 0.8 mm); **E** The callus emergence from the cotyledonary node explant of pea under 30 mg/L of PUT and 3 mg/L of 2, 4-D assisted callus induction media (bar 0.8 mm); **F** The emergence of shoot multiplication from 1.5 mg/L assisted shoot multiplication medium (bar 10 mm); **G** The emergence of shoot multiplication from 20 mg/L of SPD and 1.5 mg/L assisted shoot multiplication medium (bar 0.8 mm); **H** The shoot elongation from the 1 mg/L GA_3 shoot elongation medium (bar 1.2 cm); **I** The root induction of elongated shoots under 0.6 mg/L of NAA assisted rooting media (bar 2.5 cm); **J** The root induction of elongated shoots under 25 mg/L of PUT along with 0.6 mg/L of NAA assisted rooting media; **H** The primary hardening of in vitro regenerated plants under growth chamber environment; **H** The secondary hardening of regenerated in vitro pea plants in a greenhouse environment

Results

Plant materials

The three days of water-soaked pea (cv. Ageta 6) cotyledonary node explant (Fig. 1A, B, C) without shoot and root tips proved more adaptation and frequency of regeneration in callus induction media. It has been used as a suitable regeneration explant system for the standardization of pea's indirect organogenesis. Soaking pea seeds helps them to quickly bulge and soften, facilitating the easy removal of the seed coat and separating the seed portions. Similarly, soaking seeds makes it easier to identify healthy seeds for explant preparation since it hastens the development of the greenish seeds' shoot and root radicals.

Effect of 2, 4-D on callus induction of pea

In this study, when cotyledonary node explant were inoculated under callus induction media, they produced green, brown, and white-toned friable kinds of calluses. After the fourteenth day of explant inoculation exhibited the blooming of callus induction (Fig. 1D); from the third week onwards explant displayed the sign of shoot emergence. Among the different concentrations of 2, 4-D tested on the callus induction of pea, higher callus (green, white, and friable) induction has been recorded with maximum explant response of 11.26% (Table 1) at 3 mg/L followed by 2, 4-D at 4 mg/L, (11%) and 2 mg/l (10.66%) holds the following two positions of peas callus induction efficiency.

Effect of BA on shoot multiplication of pea

However, it was challenging to regenerate the multiple shoots formations from the calluses of pea cv. Ageta 6, 1.5 mg/L of BA exhibited relatively high shoot induction (5.36 shoots/callus piece) (Fig. 1F; Table 1) with maximum explant response (28.33%) and shoot length (1.23 cm) were achieved after three weeks. The concentration of BA at 2 and 1 mg/L have shown the second (4.53 shoots/callus) and third (3.76 shoot/callus piece) higher multiple shoot activity than the other concentrations tested.

Effect of GA_3 on shoot elongation of pea

In the present research, among the various concentration of GA_3 was tested for pea shoot elongation, 1 mg/L has shown the highest shoot elongation efficiency (6.20 cm/shoots) with the highest explant response up to 70% (Fig. 1H; Table 1).

Effect of NAA on root induction of pea

Among the different concentrations of NAA, the highest rooting (3.73 roots/shoot) with the mean root length of 1.60 cm and maximum explant response of 16.66% (Fig. 1I; Table 1) was gained from the elongated shoots at 0.6 mg/L supplemented rooting media. The higher and lower concentrations of NAA did not favor much rooting.

Effect of polyamines on callus induction

This polyamine-assisted callus induction in pea demonstrated that 30 mg/L PUT provided the highest callus induction (green, white, and friable) with maximum explant response of 46.26% (Fig. 1E; Table 2), along with the standardized concentration of 2, 4-D. Similarly, all SPD and SPM treatment concentrations produced less callus induction than at 30 mg/L of PUT.

Effect of polyamines on shoot multiplication

Among the three different polyamines of different concentrations tested in this peas shoot multiplication study, 20 mg/L of SPD has given the best shooting induction and multiplication efficiency (12.66 shoots/callus) (Fig. 1G; Table 3) with maximum explant response (53.66%) and shoot length (3.93 cm), along with the standardized concentration of BA treatment.

Effect of polyamines on root induction

Among the different concentrations of three different polyamines tested in this rooting analysis, 25 mg/L PUT along with 0.6 mg/L NAA supplemented rooting media have promoted the highest rooting efficiency up to 10.86 roots/shoot with the maximum explant response (40%) and shoot length (3.90 cm) (Fig. 1J; Table 4). The decreased or increased concentration of PUT from the standardized dose was reduced the efficiency of rooting. The exact concentration of PUT

Table 1 Effect of PGRs on callus induction, shoot multiplication, shoot elongation and root induction of pea cv. Ageta 6 from cotyledonary node explants

Concentration of 2,4-D (mg/L)	Explant response (%)	Nature of callus
1	9.43 ± 0.14e	Green-white-friable
2	10.66 ± 0.23c	Green-white-friable
3	**11.26 ± 0.17a**	Green-white-friable
4	11.00 ± 0.40b	Green-white-friable
5	10.26 ± 0.13d	Green–brown-friable
6	9.00 ± 0.12f	Brown-white-friable

Concentration of BA (mg/L)	Explant response (%)	Number of shoot per callus piece	Mean shoot length (cm)
0.5	26.66 ± 0.57e	2.00 ± 0.03e	0.40 ± 0.05e
1.0	27.33 ± 0.00c	3.76 ± 0.10c	1.00 ± 0.23c
1.5	**28.33 ± 0.33a**	**5.36 ± 0.12a**	**1.23 ± 0.05a**
2.0	27.66 ± 0.00b	4.53 ± 0.03b	1.03 ± 0.05b
2.5	27.00 ± 0.66d	3.30 ± 0.57d	0.70 ± 0.10d
3.0	26.00 ± 0.00f	1.10 ± 0.11f	0.23 ± 0.05f

Concentration of GA_3 (mg/L)	Explant response (%)	Mean shoot length (cm)
0.2	41.00 ± 0.88f	1.80 ± 0.05f
0.4	48.66 ± 0.33e	2.60 ± 0.00e
0.6	55.33 ± 0.66c	3.40 ± 0.11d
0.8	64.66 ± 0.57c	4.60 ± 0.05c
1.0	**70.00 ± 0.33a**	**6.20 ± 0.11a**
1.2	58.33 ± 0.33b	5.00 ± 0.00b

Concentration of NAA (mg/L)	Explant response (%)	Number of roots per shoot	Mean root length (cm)
0.2	15.00 ± 0.33f	1.46 ± 0.34f	0.20 ± 0.05f
0.4	15.66 ± 0.88d	2.20 ± 0.17d	0.80 ± 0.57d
0.6	**16.66 ± 0.33a**	**3.73 ± 0.24a**	**1.60 ± 0.05a**
0.8	16.33 ± 0.57b	3.03 ± 0.03b	1.20 ± 0.11b
1.0	16.00 ± 0.33c	2.73 ± 0.68c	1.03 ± 0.40c
1.2	15.33 ± 0.66e	1.26 ± 0.11e	0.65 ± 0.10e

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range tests at 5% level

(30 mg/L) and combination (PUT + NAA) has been used in our previous research, which has given the highest rooting indirect organogenesis of pea cv. Ageta 6. In comparison to PGRs alone, the Polyamine assisted callus, shoot, and root inductions were seen to quite accelerate (two to four days) regeneration efficiency.

Genetic stability analysis by RAPD and SCoT molecular markers

The regenerated plants that were successfully hardened in the greenhouse proved to be effectively acclimatized, with a survival rate of 92%. Among the 9 RAPD primers amplified against the mother pea plant DNA, there were 33 monomorphic (homologous) DNA bands (800-100 bp) were fragmented in agarose gel electrophoreses; the high scorable (4), as well as bright bands, produced RAPD primer OPD16 were selected and performed PCR amplification on eight regenerated and one mother peas genomic DNA. Even though other primers such as OPA2, OPA6, and OPA13 produced more bands (5), they were not clearly visible and scorable in this analysis; hence primer OPD16 was chosen for the molecular assessment of regenerants. The OPD16 specific RAPD investigation revealed (Fig. 2A; Table 5) no genetic instability, and conserved region mutation occurrence in the in vitro regenerated pea's DNA.

In SCoT analysis, primer S32 produced 4 stable and homogeneous morphogenic DNA fragments in agarose gel electrophoresis out of the 37 scorable monomorphic total bands (1200 to 100 bp) formed by 17 SCoT primers (Fig. 2B; Table 6) which is ultimately justified the genomic steadiness of the regenerated peas without somoclonal differences.

Table 2 Effect of polyamines on callus induction of pea cv. Ageta 6 from cotyledonary node explants

Concentration of PAs (mg/L)		Concentration of 2,4-D (mg/L)	Explant response (%)	Nature of callus
SPD	5	3	12.56 ± 0.13w	Brown-white-friable
	10	3	14.03 ± 0.14u	Brown-white-friable
	15	3	23.03 ± 0.14j	Green-white-friable
	20	3	33.26 ± 0.40d	Brown-white-friable
	25	3	28.43 ± 0.12g	Green-white-friable
	30	3	20.56 ± 0.13m	Green-white-friable
	35	3	18.66 ± 0.23p	Green-white-friable
	40	3	16.03 ± 0.14s	Green-white-friable
SPM	5	3	11.56 ± 0.17x	Brown-white-friable
	10	3	12.56 ± 0.17v	Brown-white-friable
	15	3	21.66 ± 0.23k	Green-white-friable
	20	3	30.66 ± 0.23e	Brown-white-friable
	25	3	25.56 ± 0.13h	Green-white-friable
	30	3	19.56 ± 0.17n	Green–brown-friable
	35	3	16.43 ± 0.12q	Green-white-friable
	40	3	14.56 ± 0.13t	brown-white-friable
PUT	5	3	16.33 ± 0.14r	Green-white-friable
	10	3	19.43 ± 0.12o	Green-white-friable
	15	3	21.56 ± 0.13l	Brown-white-friable
	20	3	28.73 ± 0.17f	Green–brown-friable
	25	3	39.73 ± 0.17b	Green–brown-friable
	30	**3**	**46.26 ± 0.40a**	Green-white-friable
	35	3	37.56 ± 0.13c	Green-white-friable
	40	3	24.73 ± 0.17i	Green–brown-friable

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range tests at 5% level

Photosynthetic, chloroplast, and antioxidant analysis

The enhanced photosynthetic profile, including chlorophyll a (549.67 µg/g of FW), chlorophyll b (331.39 µg/g of FW), and carotenoid (130.14 µg/g of FW) outcome were recorded (Fig. 2E) in this study by the supplement of polyamines SPD (20 mg/L) and PUT (25 mg/L) assisted shooting and rooting pea regenerants compared with photosynthetic ranges of control plants such as chlorophyll-a (104.75 µg/g of FW), chlorophyll b (80.15 µg/g of FW) and carotenoid (33.56 µg/g of FW). This polyamine-assisted pea indirect regeneration study understood approximately four-fold improved total chlorophyll content in polyamine treated pea regenerants.

Interestingly, in the confocal microscopic visualization of chloroplast, the precise and improved chloroplast arrangement was captured (Fig. 2D) in the cells of polyamine-assisted pea regenerants than the cells of PGR assisted pea regenerants without polyamine supplement (Fig. 2C). The present study exhibited enhanced antioxidant activity in polyamine-assisted pea regenerants than control plants. The H_2O_2, DPPH, and NO scavenging assays were raised to 68.95, 70.86, and 65.29% in 100 µg/ml of polyamines SPD (20 mg/L) and PUT (25 mg/L) assisted plant samples than control plant (25.67, 20.40, and 20.50%) (Fig. 2F). In comparison to all other samples tested, the standard (Ascorbic acid) had the highest radical scavenging activity in all the three antioxidant assays.

Discussion

Effective pea shoot emergences and multiplication can be possible in cotyledonary node explant due to the efficient pre-existing meristems and active *de nova* dividing responses and promoting rapid morphogenic differentiation (Duclercq et al., 2011; Jackson & Hobbs, 1990). Jackson and Hobbs (1990) study suggests that the cotyledonary node has produced maximum buds and shoot formation (7.4 and 8.6) among three explant systems (cotyledonary node, immature leaflet, and plumule) tested in the two different pea genotypes. Furthermore, our previous study (Ajithan et al., 2019) on the direct organogenesis of peas cv. Ageta 6 with the help of polyamines yielded positive outcomes on the cotyledonary node explant system, which demonstrating the suitability of the cotyledonary node explant for stable and reliable pea regeneration. Jordan and Hobbs (1993) and Svabova and Griga (2008) have also used cotyledonary node explant to standardize the effective *Agrobacterium*-mediated pea genetic transformation studies.

The callus is an undifferentiated totipotent cell mass from the differentiated cells that make up the entire plant body. In the in vitro state, there is an immediate callus-induction at a balanced concentration of exogenous auxins and cytokines supplement; 2, 4-D is a vital synthetic auxin that is actively involved in the induction of callus in both dicot and monocot plants (Steward et al., 1958; Nagata & Takebe, 1970; Yamada, 1993; Naqvi et al., 2002). Notable pea callus induction was established by some researchers using 2, 4-D at higher and even lower than the concentration we recorded; The higher concentration of 2, 4-D was utilized by Bailey (1970), who applied 6 mg/L 2, 4-D for the pea's pisatin production study through callus induction. Bala et al., (2010) derived pea callus from 5 mg/L of 2, 4-D enriched callus induction media. Some of the lower concentrations of 2, 4-D may also be reported for pea callus induction; reported by Hashimoto et al., (1989), who utilized 0.1 mg/l of 2,

Table 3 Effect of polyamines on shoot multiplication of pea cv. Ageta 6 from callus pieces (explants)

Concentration of PGRs (mg/L)		Concentration of BA (mg/L)	% of explants response	Number of shoot per callus piece	Mean shoot length (cm)
SPD	5	1.5	38.00 ± 0.00l	7.36 ± 0.60l	1.50 ± 0.05l
	10	1.5	40.00 ± 0.57i	8.60 ± 0.03i	2.00 ± 0.11i
	15	1.5	47.00 ± 0.00c	11.10 ± 0.33c	2.66 ± 0.05c
	20	**1.5**	**53.66 ± 0.33a**	**12.66 ± 0.64a**	**3.93 ± 0.05a**
	25	1.5	48.66 ± 0.57b	11.30 ± 0.44b	2.70 ± 0.05b
	30	1.5	42.30 ± 0.33f	10.10 ± 0.04f	2.23 ± 0.17f
SPM	5	1.5	43.33 ± 0.33e	6.23 ± 0.12q	0.93 ± 0.03q
	10	1.5	37.30 ± 0.00m	7.43 ± 0.22m	1.33 ± 0.11m
	15	1.5	38.66 ± 0.33j	8.03 ± 0.08j	1.70 ± 0.05j
	20	1.5	45.66 ± 0.57d	10.26 ± 0.08d	2.40 ± 0.05d
	25	1.5	42.00 ± 0.66g	9.26 ± 0.96g	2.13 ± 0.17g
	30	1.5	35.33 ± 0.33o	7.13 ± 0.70o	1.13 ± 0.03o
PUT	5	1.5	28.33 ± 0.00l	5.40 ± 0.55r	0.36 ± 0.20r
	10	1.5	33.30 ± 0.00p	6.60 ± 0.96p	1.00 ± 0.05p
	15	1.5	36.66 ± 0.57n	7.03 ± 0.06n	1.26 ± 0.05n
	20	1.5	38.33 ± 0.66k	8.36 ± 0.78k	1.63 ± 0.05k
	25	1.5	43.33 ± 0.33e	10.03 ± 0.36e	2.30 ± 0.11e
	30	1.5	41.30 ± 0.57h	9.06 ± 0.18h	2.03 ± 0.20h

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range tests at 5% level

Table 4 Effect of polyamines on root induction of pea cv. Ageta 6 from elongated shoots (explant)

Concentration of PGRs (mg/L)		Concentration of NAA (mg/L)	% of shoots produced roots	Number of roots per shoot	Mean root length (cm)
SPD	5	0.6	18.33 ± 0.33r	4.13 ± 0.34r	0.40 ± 0.00r
	10	0.6	21.66 ± 0.66p	5.13 ± 0.03p	0.83 ± 0.17p
	15	0.6	28.66 ± 0.33j	6.96 ± 0.03j	2.00 ± 0.05j
	20	0.6	35.33 ± 0.66d	8.50 ± 0.31d	3.13 ± 0.05d
	25	0.6	31.33 ± 0.33g	7.56 ± 0.06g	2.73 ± 0.11g
	30	0.6	26.33 ± 0.57m	6.06 ± 0.03m	1.20 ± 0.11m
SPM	5	0.6	16.66 ± 0.33s	3.20 ± 0.63s	0.20 ± 0.05s
	10	0.6	19.66 ± 0.33q	4.73 ± 0.42q	0.53 ± 0.00q
	15	0.6	27.33 ± 0.57k	6.70 ± 0.03k	1.93 ± 0.05k
	20	0.6	32.66 ± 0.33e	8.33 ± 0.06e	3.06 ± 0.11e
	25	0.6	31.00 ± 0.00h	7.30 ± 0.67h	2.20 ± 0.20h
	30	0.6	25.66 ± 0.57n	5.66 ± 0.49n	1.13 ± 0.05n
PUT	5	0.6	23.33 ± 0.66o	5.40 ± 0.26o	1.00 ± 0.17o
	10	0.6	26.66 ± 0.33l	6.16 ± 0.86l	1.46 ± 0.05l
	15	0.6	30.33 ± 0.00i	7.10 ± 0.00i	2.08 ± 0.05i
	20	0.6	36.33 ± 0.57b	9.66 ± 0.03b	3.50 ± 0.05b
	25	**0.6**	**40.00 ± 0.00a**	**10.86 ± 0.42a**	**3.90 ± 0.05a**
	30	0.6	35.66 ± 0.00c	9.23 ± 0.03c	3.20 ± 0.20c
	30	0.6	32.33 ± 0.33f	8.17 ± 0.17f	3.00 ± 0.03f

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range tests at 5% level

4-D to raise callus induction along with 0.5 mg/l of kinetin. Puonti-Kaerlas et al., (1990) have found that 0.5 mg/L of 2, 4-D has produced successful callus induction along with 0.5 mg/L BA. In line with our result, Lulsdorf et al., (1991) used 2 mg/L of 2, 4-D to achieve the callus induction and the same concentration of BA in their transgenic experiments. Olmos et al., (1994) has raised callus culture from 1 mg/L 2, 4-D media for the antioxidant analysis in salt-tolerant peas.

Cytokinins are the dominant cluster of plant hormones primarily involves plant cell division and development. Benzyladenine is the first, and the effective synthetic cytokinins efficiently stimulate plant regeneration and tissue morphogenesis (Koshimizu & Iwamura, 1986). Previous studies have validated the application of low and high concentration BA to induce shooting from pea callus; Gamborg et al., (1974) reported that 0.45 mg/L BA caused the most shoot formation (4 shoots/callus piece) from pea callus. Natali and Cavallini (1987) have used 0.5 mg/L BA and 0.2 mg/L NAA to induce callus induction and shoot development in pea. Hussey and Gunn (1984) have produced shoot multiplication from the combination of BA (1 mg/L) and IBA (0.25 mg/L) supplement.

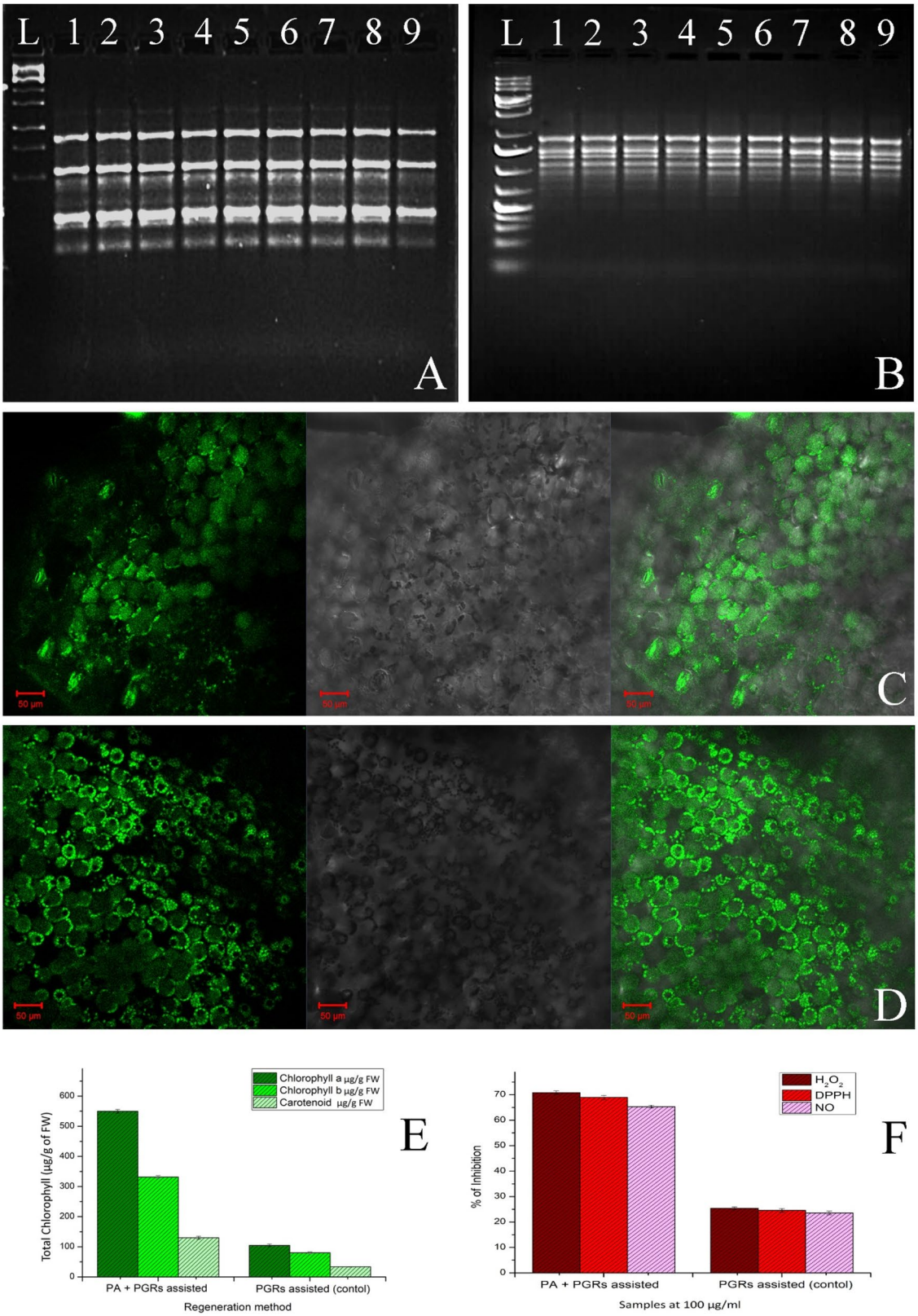
In line with our outcome, Grant et al., (1995) have used B5 medium, which supplemented with 1.3 mg/L BA for both calli as well as shoot induction to produce efficient transgenic pea lines; Sharma et al., (2017) suggested MS media supplemented with 4.50 mg/L BA with 1.86 mg/L NAA shooting media to reach the optimal shoot regeneration from the hypocotyl explant of a pea. Higher concentrations of BA (5 mg/L) with IAA (0.2 mg/L) have been administered by Malmberg, (1979) to produce shoot multiplication from the callus pieces of pea explant. Schroeder et al., (1993) used 4.5 mg/L BA and 0.02 mg/L NAA for the shoot multiplication of pea from callus culture. Similarly, Puonti-Kaerlas et al. (1990) successfully produced callus induction and shoot multiplication from the epicotyl explants of pea using 5 mg/L BA, the same concentration of Kinetin, and 1 mg/L of abscisic acid (ABA).

Shoot elongation is a crucial morphogenic differentiation in higher plants; it allows leaves to connect directly with atmospheric oxygen above the soil or water surface, which boosts the rate of photosynthesis (Voesenek et al., 2004). Rapid in vitro shoot, internode elongation, and cell numbers can be achieved by the exogenous GA_3 treatment (Kato et al., 2011; Little & Macdonald, 2003; Shan et al., 2021; Srivastava & Handa, 2005). Elongation could be aided by gibberellic acid that enhances either wall extensibility or turgor pressure. Gibberellic acid attributed plant wall extensibility might be occurred by directing the up or down-regulation of wall tightening and loosening process and wall polysaccharides synthesis (Cleland, 1981; Cosgrove & Sovonick-dunford, 1989; Fry, 1980; Montague & Ikuma, 1975; Nakamura et al., 1975; Stuart & Jones, 1977). Das et al., (2014) used the 1 mg/L GA_3 along with 2 mg/L BA and 0.4 mg/L NAA hormone combination to induce 85% shoot elongation in pea plants. Our previous direct organogenesis standardization in pea cv. Ageta 6 was also yielded similar results (Ajithan et al., 2019).

In line with our result, Sharma et al., (1996) recorded the maximum root induction (93.5%) on the shoots of pea cv. Arkel under 0.5 mg/L NAA enriched rooting medium. It's worth noting that in our previous direct organogenesis experiment (Ajithan et al., 2019) in peas cv. Ageta 6, a similar dose of NAA resulted in progressive root development. Many researchers have utilized 1 mg/L NAA to increase the success of pea root induction; for example, Malmberg, (1979) employed 1 mg/L NAA for the root induction of pea by shoots dipping in NAA for 10 s. According to Das et al., (2014), 1 mg/L NAA can increase pea genotype IPF 4–26 rooting frequency up to 55–60%. Under 1 mg/L NAA augmented half-strength MS root induction medium, Ochatt et al., (2000b) began pea rooting from microcalluses. Puonti-Kaerlas et al., (1990) employed 0.19 mg/L NAA and 0.8 mg/L IAA for root induction of pea shoots, which contradicted our findings.

Polyamine is an aliphatic amine that plays a key activity in plant growth and development, particularly callus induction and morphogenesis. Several studies have shown that endogenous polyamines (PUT) in plants rise during callus initiation and growth, and exogenously supplementing polyamines (PUT) has significantly aided the callus induction organogenesis process (Debiasi et al., 2007; Koetje et al., 1993; Mógor et al., 2007; Takeda et al., 2002; Viu et al., 2009). Rajesh et al., (2003) used a similar PGR and PA composition (2, 4-D at 0.01 mg/L and PUT at 0.08 mg/L) to create the highest embryogenic calluses and somatic embryos in oil palm. Viu et al., (2009) has proposed a 1:1:1 ratio of PUT, SPD, and SPM at 5 or 10 mM/L supplemented growth media combined with 2 mg/L NAA to produce maximum callus induction from the bud explant of Turmeric. Tang et al., (2004) reported 0.13 mg/L of PUT supplemented TE medium along with BA (0.5 mg/L), and IAA (2 mg/L) has rejuvenated the maximum number of pine browning tissues to typical callus culture. Some researchers have justified using 2, 4-D and PUT combined induced improved callus induction in plants.

Polyamines are the natural substances that can promote the rapid proliferation and multiplication of plants by delivering stable carbon and nitrogen sources and act as secondary messengers that inhibit plant growth (Couée et al., 2004; El Ghachtouli et al., 1996; Martin-Tanguy, 2001; Purohit et al., 2007; Sivanandhan et al., 2011). A similar combination of SPD (20 mg/L) with BA (1.5 mg/L) has produced maximum multiple shooting in pea cv. Ageta 6 in our direct organogenesis experiment (Ajithan et al., 2019). Vasudevan et al., (2017) also used the same combination of SPD


L 1 2 3 4 5 6 7 8 9
A
L 1 2 3 4 5 6 7 8 9
B
50 µm
C
50 µm
D
Chlorophyll a µg/g FW
Chlorophyll b µg/g FW
Carotenoid µg/g FW
Total Chlorophyll (µg/g of FW)
0
100
200
300
400
500
600
PA + PGRs assisted
PGRs assisted (contol)
Regeneration method
E
H2O2
DPPH
NO
% of Inhibition
0
10
20
30
40
50
60
70
PA + PGRs assisted
PGRs assisted (contol)
Samples at 100 µg/ml
F

◂**Fig. 2** **A** RAPD analysis of in vitro regenerated and mother plant with primer OPD16. *lane L* 1 kb plus DNA ladder; *lanes 1–8* in vitro regenerated plant DNA; *lane 9* mother plant DNA; **B** SCoT analysis of in vitro regenerated and mother plant DNA with primer S32. *lane L* 1 kb DNA ladder; *lanes 1–8* in vitro regenerated plants DNA; *lane 9* mother plant DNA; **C** Confocal microscopic visualization of chloroplasts in control plant; **D** confocal microscopic visualization of chloroplast in polyamine assisted in vitro regenerated plants; **E** Quantification of photosynthetic pigments in vitro regenerated and control plant; **F** Antioxidant (H_2O_2, DPPH and NO) analysis of in vitro regenerated and control plants

(10 mg/L) along with BA (1 mg/L) to produce maximum shoots in the watermelon. Sivanandhan et al., (2011) suggested 20 mg/L SPD-assisted shooting media and t 1.5 mg/L BA and 0.3 mg/L IAA to achieve maximum multiple shooting in ashwagandha.

Putrescine is one of the critical polyamines involved in effective control of ethylene production and raising plant morphogenesis, especially in primary, lateral, and adventitious root induction (Bais & Sudha 2000; Nas, 2004; Couée et al., 2004). Vasudevan et al., (2017) reported the highest rooting efficiency in watermelon under 10 mg/L PUT assisted rooting media along with 1 mg/L IBA.

Inplant tissue culture technique somaclonal variation is the unexpected dissimilarity that occurs in a natural genetic pattern of the plants, resulting in mutated offspring, these genomic abnormalities can effectively be pointed out by DNA-based molecular marker technique RAPD using random primers. In our previous direct organogenesis research (Ajithan et al., 2019), we used the same RAPD primer (OPD16) for the examination of genetic originality of the in vitro regenerated pea plants.

Each gene expression begins from the start or initiation codon, which is significantly crucial for the respective protein synthesis and biological functions. The practice of diagnosing new generation genetic troubles from a start codon is evolving a potent genetic technology. The mutations or deviations in the common start codon (ATG) of the genes of an organism can be quickly and effectively witnessed by "start codon targeted polymorphism" molecular investigation (Collard & Mackill, 2009). Although SCoT molecular markers were assessed in series of plants like (Amirmoradi et al., 2012), common wheat and rice (Collard & Mackill, 2009), sugarcane (Sathish et al., 2018), chickpea (Hamidi et al., 2014), common grape (Guo et al., 2012) and watermelon (Vasudevan et al., 2017) for the countless analysis like cultivar recognition, mapping quantitative trait loci (QTL), DNA fingerprinting and Genetic fidelity analysis, it has not yet been widely employed in the genetic composure experiments in pea except our previous peas direct organogenesis study (Ajithan et al., 2019). This study has also given a similar positive amplification by applying the same SCoT primer (S32).

The exogenous polyamine can boost the chloroplast apparatus (PSII) system by binding with negatively charged photosynthetic proteins and stabilizing the membranes of thylakoids and light-harvesting complexes (LHC) which facilitate the improved chloroplast count and chlorophyll content in the plant system (Ajithan et al., 2019; Baryla et al., 2001; Galston et al., 1997; Kakkar & Nagar, 1996; Kaur-sawhney & Galston, 1979; Lee et al., 1997; Shu et al., 2012; Zhang et al., 2009). The polyamine (PUT) prevented membrane degradation in the granal and stromal thylakoids under salinity stress (Tiburcio et al., 1994). This result obtained in this study was consistent with the result revealed in our previous study (Ajithan et al., 2019), which demonstrates that polyamines increase the chloroplast number and chlorophyll level in peas. Vasudevan et al., (2017) found that polyamine treated in vitro watermelon plants had five-fold increased chlorophyll content. The dense network and envelop of the thylakoid lamellae expanded with several stacked grana after exogenous SPD assistance on *Brassica campestris* leaf disc (Pjon et al., 1990). Exogenous spermidine treatment increased chlorophyll production and net photosynthetic rate in cucumbers, according to the research of Shu et al., (2012).

Exogenous polyamines promote photosynthesis, neutralize the oxidative stress damages, and improve the intact chloroplast structure and CO_2 fixation by preventing the degradation of stroma-localized protein rubisco. They also improve scavenges of ROS free radicals like DPPH, H_2O_2, and NO via increased catalase activity, reduced lipid peroxidation, membrane leakage, and lowered sodium/potassium ratio (Drolet et al., 1986; Gill et al., 2010; Hassan et al., 2020). Similar enhanced antioxidant profiles were noted in gherkin (Thiruvengadam & Chung, 2015), rock cress (Tun et al., 2006) under exogenous polyamine supplements. Same way, the elevated antioxidant profile has been found in regenerated pea cv. Ageta 6 by Ajithan et al., (2019).

Conclusion

The successful tissue culture technique for the recalcitrant Indian pea cv. Ageta 6 was developed by using polyamine-assisted indirect organogenesis approach, which improved callus induction, shoot multiplication, and root induction of pea regenerants with no somaclonal variation, quadrupled chlorophyll, tripled antioxidant levels, and significantly increased the number of chloroplasts. The peas regenerated in this PA-aided regeneration system have a high probability of surviving even in intense oxidative stress conditions due to their increased photosynthetic and antioxidant characteristics. In addition, our clonal polymorphism-free rapid pea regeneration technique could be a viable tool for large-scale genetic transformation investigations in pea against a variety of biotic and abiotic challenges.

Table 5 List of RAPD primers and their sequences, number and size of the amplified fragments generated in the DNA of pea cv. Ageta 6

No	Primer Name	Primer sequence (5′–3′)	Number of scorable monomorphic bands	Size range of bands (bp)
1	OPA2	TGCCGACCTG	5	800–200
2	OPA6	GGTCCCTGAC	5	500–100
3	OPA7	GAAACGGGTG	3	400–200
4	OPA8	GTGACGTAGG	4	600–200
5	OPA11	CAATCGCCGT	2	200–100
6	OPA13	CAGCACCCAC	5	700–100
7	OPA14	CTCGTGCTGG	3	600–100
8	OPD13	GGGGTGACGA	2	400–100
9	OPD16	AGGGCGTAAG	4	500–100
Total			33	800–100

Table 6 List of SCoT primers and their sequences, number and size of the amplified fragments generated in the DNA of pea cv. Ageta 6

No	Primer name	Primer sequence (5′–3′)	Number of scorable monomorphic bands	Size range of bands (bp)
1	S1	CAACAATGGCTACCACCA	1	100
2	S2	CAACAATGGCTACCACCC	1	400
3	S3	CAACAATGGCTACCACCG	2	500–300
4	S4	CAACAATGGCTACCACCT	3	400–300
5	S5	CAACAATGGCTACCACGA	2	400–300
6	S6	CAACAATGGCTACCACGC	2	500–300
7	S7	CAACAATGGCTACCACGG	1	300
8	S10	CAACAATGGCTACCAGCC	2	800–500
9	S11	AAGCAATGGCTACCACCA	2	500–200
10	S12	ACGACATGGCGACCAACG	3	400–100
11	S16	ACCATGGCTACCACCGAC	3	700–200
12	S17	ACCATGGCTACCACCGAG	1	1200
13	S25	ACCATGGCTACCACCGGG	3	300–100
14	S26	ACCATGGCTACCACCGTC	1	600
15	S32	CCATGGCTACCACCGCAC	4	500–100
16	S34	ACCATGGCTACCACCGCA	3	500–300
17	S36	GCAACAATGGCTACCACC	3	500–100
Total			37	1200–100

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Author contributions CA: Designing and executing the work; VV: Data correction; SS and GP: Formal analysis; EY: Visualization; MM: Conceptualization, Investigation and Supervision.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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