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The fascinating role of plant growth regulators (PGRs) on the callus dependent indirect regeneration of Indian pea (*Pisum sativum* L.) cv. Arka Sampoorne

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ABSTRACT
The pea plant is one of the most essential legumes in the world due to its excellent protein content. The pea plant, which is an excellent alternative to animal protein, is a naturally recalcitrant crop plant due to its unique genetic makeup. This study is set to illustrate the positive properties of the plant growth regulators (PGRs) in the callus dependent indirect tissue culture of the recalcitrant Indian pea cultivar ArkaSampoorne. The cotyledonary node was used as an explant system for producing pea calluses. This study looked at the effects of the callus induction, shoot multiplication, and rooting stages of pea regeneration. Among the different 2,4-D concentrations tested, 3 mg/L produced the highest explant response on callus induction of up to 9.0% with green-white-friable calluses. In shoot multiplication studies, 1.5mg/L of BA produced a 4.0 shoot per explant with a 1 cm mean shoot length and a 27.0% explant response. In the shoot elongation studies, the maximum shoot elongation was found at 6.0 cm shoot length with a 67.0% explant response at 1 mg/L of GA_3 assistance. The standardized indirect organogenesis protocol appears to be promising for achieving stable regeneration and tissue culture-based genetic manipulation of the Indian pea cv. ArkaSampoorne.
Keywords: *ArkaSampoorne, Callus, Indirect organogenesis, Pea, Plant growth regulators, Rooting, Shooting, Tissue culture*

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INTRODUCTION

Plant tissue culture, which is nothing but the aseptic culturing of plant cells, tissues or organs under systematic chemical and physical circumstances, is a vitally needed technique in basic, applied and also commercial research and application [39]. The use of plant tissue culture practices in single-cell to embryo systems allows the rejuvenation of recalcitrant legumes [26]. Despite the high genotype dependency and recalcitrant nature of legumes like peas experiencing troubles in *in vitro* culturing like organogenesis, stable and reproducible indirect regeneration and callus culture provide promising upliftment for the advanced regeneration of numerous grain legumes like chickpea, soybean, pigeon pea, green pea, cowpea, and mungbean towards genetic manipulation to create additional positive genetic variation [9, 29].

The pea is a high-protein, herbaceous, recalcitrant annual crop in the legume family (Fabaceae) that is cultivated as an edible vegetable all over the world. Pea production in the world has risen dramatically from human and cattle consumption, reaching 19.8 million tones by the year 2020. Hildebrandt *et al.*, [12] created the first pea regeneration process by obtaining pea callus from a stem explant. Although many scientists, including Bailey [2], Hashimoto *et al.,* [11], Kosturkova *et al.*, [18], Puonti-Kaerlas *et al.*, [30], Lulsdorf *et al.* [20], Olmos *et al.*, [28]; Bala *et al.*, [3], have worked on callus dependent tissue culture events of a pea, there is a strong need to identify new tissue culture methods for every cultivar of grain legume, such as pea, that has been grown across the world [1, 27, 29]. From the above understanding, this study is designed to focus on the indirect organogenesis of the well-known recalcitrant Indian pea cultivar ArkaSampoorne with the assistance of different plant growth regulators.

MATERIAL AND METHODS

Plant materials and explant preparation

ArkaSampoorne, a popular Indian pea cultivar, was purchased from the Indian Institute of Horticulture Research (IIHR) in Bangalore, Karnataka, India, and used to standardise the indirect organogenesis of pea experiments. The seeds were disinfected using Di *et al*., [6] and Ajithan *et al*., [1] recommended chlorination method of sterilization. The sterilized seed was soaked in sterile water for three days before being processed for explants. The full-strength MS [22] media has been used for the preparation of all growth media such as callus induction (CIM), shoot multiplication (SIM), shoot elongation (SEM) and root induction media (RIM) with the addition of 8% solidification agent Agar Agar. The media were sterilized using the steam autoclave method at 110 kPa (121 °C) for 30 minutes. All the needed chemicals and plant growth regulators were purchased from HiMedia®, Mumbai, India.

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

The callus induction experiment was performed by inoculating the sterilized and dissected pea explants with different doses (1–6 mg/L) of 2,4-D added CIM for 3 weeks. The shoot multiplication studies were performed by inoculating the callus pieces to the SIM, which constituted different doses (0.5–3.0 mg/L) of BA for 3 weeks. The shoot elongation studies were done after successful shoot multiplication. The multiplied shoots were subjected to SEM inoculation consisting of different concentrations (0.2-1.2 mg/L) of GA_3 for 3 weeks. The rooting studies were done using RIM media, which consists of different doses (0.2-1.2 mg/L) of NAA for 4 weeks. All studies were carried out at 25±2 °C with a 16–8-hour cool fluorescent photoperiod with 50 mol m-2 s-1 irradiance.

Hardening and acclimatization

Hardening of regenerated pea plants was accomplished by inoculating thoroughly washed regenerants in a paper cup of soil mix (1:1:1 v/v/v ratio of soil, sand, and soil rite) under growth chamber conditions for two weeks before moving to a large pot of soil mix under greenhouse conditions for two weeks. In a growth chamber, all of the plants were kept at a moisture level of 80%. The humidity for plants was maintained by plastic bag covering of regenerants for two weeks and then moved to greenhouse acclimatization without plastic covers.

Statistical analysis

Each experiment was carried out three times with 100 explants per treatment. The Duncan Multiple Range Test and one-way ANOVA were used to analyse the data (DMRT). For statistical analysis (with a P value less than 0.05), SPSS 20 (SPSS Inc, Armonk, New York, USA) was used, and graphs were created with Origin (OriginPro 8, MicroCalInc, Westborough, Massachusetts, USA) on Windows 8.0.

RESULTS AND DISCUSSION

Plant materials

The cotyledonary node explant method is an excellent choice for pea regeneration since it allows for fast morphogenic differentiation [7, 14, 15]. The notable pea regeneration works have been published by handling cotyledonary node explants as a suitable explant system by Jackson and Hobbs [14]; Jordan and Hobbs [15]; Svabova and Griga [38]; Ajithan *et al.,* [1]. In this study, the development of an appropriate explant system initiated the indirect regeneration of Indian pea cv. ArkaSampoorne (Fig. 1A). The hypocotyl shoot tip, epicotyl root tip, and two half cotyledons excised cotyledonary node explant (Fig. 1B) were chosen as an appropriate explant system for emerging pea regenerants via this callus mediated indirect regeneration.

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

Synthetic plant growth hormone 2,4-D can trigger undifferentiated cell mass induction in both dicot and monocot plants [36, 23, 24, 41]. The 2,4-D assisted pea callus induction has been published by Bailey [2]; Bala *et al.,* [3]; Hashimoto *et al*., [11], Puonti-Kaerlas *et al*., [30]; Lulsdorf *et al*., [20]; Olmos *et al.,* [28]. In this study, the highest explant response up to 9.0 % (Fig. 1C; Table 1) towards callus initiation in ArkaSampoorne was found at 3mg/L of 2,4-D in the varied concentrations of 2,4-D induced callus induction experiment. After the second week of CIM inoculation, the callus was observed as green-white and friable. The second and third highest callus induction efficiency was found in 4mg/L (8.66%) and 2mg/L (8.26%) 2, 4-D assistance, which yielded green-white and friable calluses, respectively.

Effect of BA on shoot multiplication of pea

Synthetic cytokinin BA or BAP, an ultimate plant growth hormone that induces multiple shoots in pea, can greatly influence plant morphology and regeneration [17,]. Commendable regeneration experiments in pea have been published by utilizing BA as an effective shoot multiplication inducer by Gamborg *et al*., [8]; Natali and Cavallini [25]; Hussey and Gunn [13]; Grant *et al*., [10]; Sharma *et al*., [33]; Malmberg, [21]; Schroeder *et al*., [31]; Puonti-Kaerlas *et al*., [30]; Ajithan *et al.,* [1]. In this study, the multiple shooting study of the callus pieces of ArkaSampoorne displayed the highest shoot multiplication phase at 1.5 mg/L

BA (Fig. 1D; Table 1). This concentration of BA has produced 4.0 number of 1cm long shoots per explant with the highest explant response of 27.0% compared with other concentrations of BA. The second and third highest shoot multiplication have been found in 2mg/L and 1mg/L of BA assistance, which has raised 0.7cm shoot length of 3.30 and 2.76 number of shoots per explant with 26.33% and 26.00% of explant response, respectively.

Effect of GA_3 on Shoot elongation of pea

Due to its rapid cell wall extensibility property, GA_3 can effectively be involved in the shoot elongation process of vast plant species [4, 19]; Srivastava and Handa, [35]; Kato *et al*., [16]; Shan *et al*., [32]; Das *et al*., [5]; Ajithan *et al*., [1] successfully use GA_3as an appropriate PGR for improving shoot elongation of pea plants. Among the different doses of GA_3 evaluated for the pea shoot elongation experiment in this study, 1 mg/L exhibited the maximum shoot elongation efficiency (Fig. 1E; Table 1) with 6.0 cm of shoot elongation per shoot with the maximum explant response (67.0%). The second and third highest shoot elongation were recorded in 0.8 (4.6 cm) and 1.2 mg/L (4.0 cm) of $GA_3$with explant responses of 60.6% and 56.3% respectively.

Effect of NAA on root induction of pea

A little amount of synthetic auxin NAA can efficiently stimulate the root induction pathway of the plant system by enhancing cell growth and division strategies [37]. Some of the significant root induction studies done in pea by using NAA as a suitable PGR, Sharma *et al.,* [33]; Ajithan *et al*., [1] Malmberg, [21]; Das *et al*., [5]; Ochatt *et al*., [27]; Puonti-Kaerlas *et al*., [30]. The root induction experiment revealed the maximum root induction in ArkaSampoorne elongated shoots under 0.6mg/L NAA assisted SEM (Fig. 1F; Table 1). This dose of NAA has produced 3.20 roots per explant with 1.2cm root length along with 15.66% explant response. The second and third highest rooting efficiency with 1cm of root length has been noted in 0.8mg/L (2.73 numbers of roots per shoot) and 1mg/L (2.20 number of roots per shoot) of NAA assisted RIM with 15.33% and 14.88% explant response respectively. The healthy rooted plants were hardened (Fig. 1G) and then transferred to pot (Fig. 1H) with the success rate of 64%.

Table 1 The PGRs assisted callus induction, shoot multiplication, shoot elongation and root induction studies on the cotyledonary node explant of pea cv. ArkaSampoorne

Concentration of 2,4-D (mg/L)	**Explant response (%)**	**Nature of Callus**	
1	7.66±0.23e	Green-White-Friable	
2	8.26±0.40c	Green-White-Friable	
3	**9.00±0.40a**	**Green-White-Friable**	
4	8.66±0.40b	Green-White-Friable	
5	8.00±0.23c	Green-Brown-Friable	
6	7.00±0.23e	Green-Brown-Friable	
Concentration of BA (mg/L)	**Explant response (%)**	**Number of shoot per callus piece**	**Mean shoot length (cm)**
0.5	25.00±0.33e	1.30±0.03e	0.40±0.00e
1.0	26.00±0.00c	2.76±0.10c	0.70±0.00c
1.5	**27.00±0.00a**	**4.00±0.12a**	**1.00±0.23a**
2.0	26.33±0.66b	3.30±0.03b	0.70±0.23b
2.5	26.33±0.66d	2.30±0.12d	0.40±0.23d
3.0	25.00±0.00f	1.00±0.12f	0.10±0.23f
Concentration of GA_3 (mg/L)	**Explant response (%)**	**Mean shoot length (cm)**	
0.2	40.33±0.00f	1.60±0.05f	
0.4	46.33±0.33e	2.40±0.11e	
0.6	53.00±0.66d	3.00±0.11d	
0.8	60.66±0.33b	4.60±0.11b	
1.0	**67.00±0.66a**	**6.00±0.11a**	
1.2	56.33±0.66c	4.00±0.11c	
Concentration of NAA (mg/L)	**Explant response (%)**	**Number of roots per shoot**	**Mean root length (cm)**
0.2	14.00±0.00f	0.76±0.11f	0.20±0.00f
0.4	14.66±0.88d	1.73±0.13d	0.80±0.20d
0.6	**15.66±0.00a**	**3.20±0.17a**	**1.20±0.40a**
0.8	15.33±0.57b	2.73±0.03b	1.00±0.57b
1.0	14.88±0.33c	2.20±0.68c	1.00±0.10c
1.2	14.00±0.66e	1.00±0.13e	0.40±0.57e

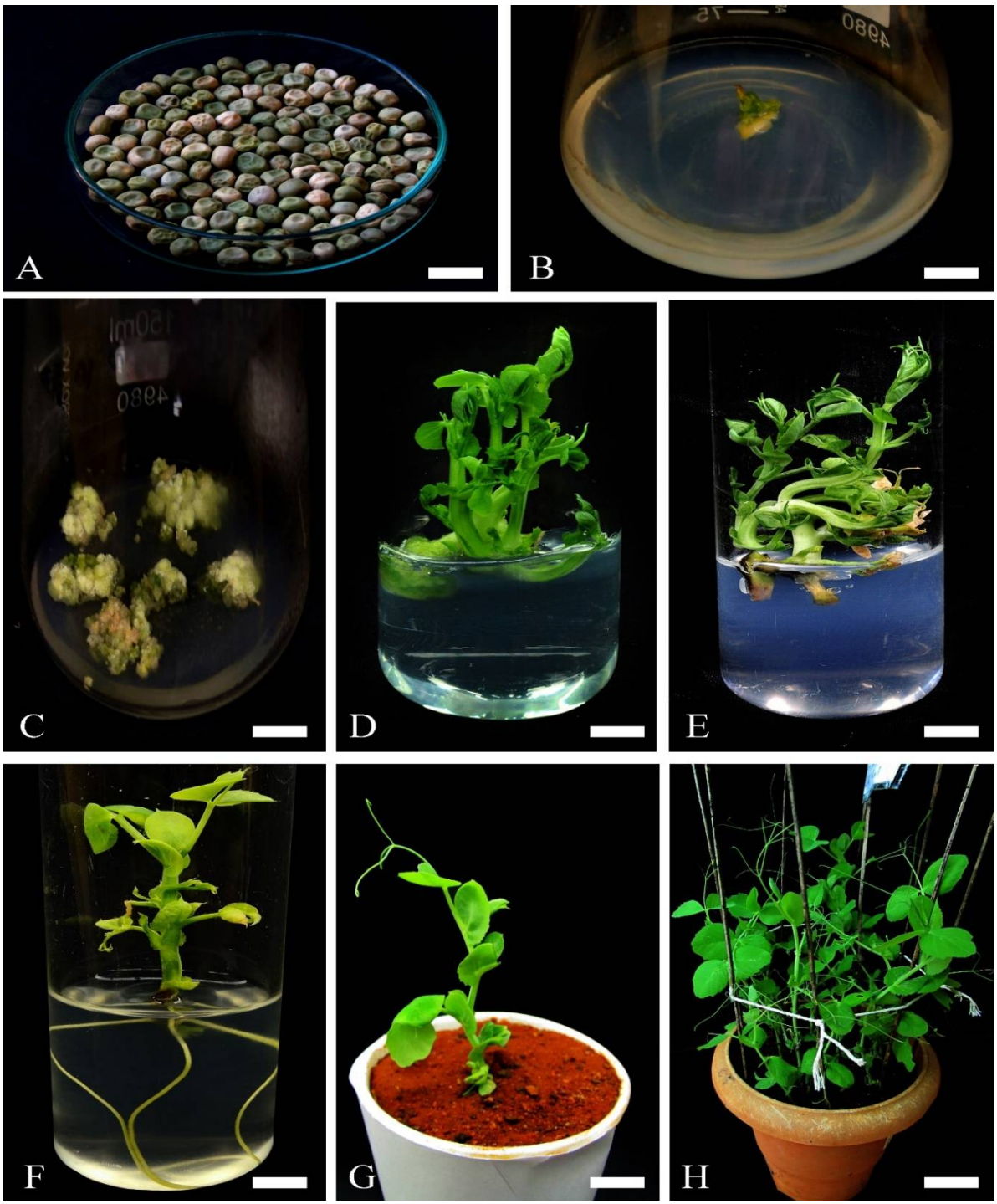

Fig 1, A pea seeds (cv. ArkaSampoonre) used for regeneration studies (bar 1 cm); **B** The cotyledonary node explant inoculated in callus induction media (bar 0.3 mm); **C** The callus induction from the inoculated explant of pea (bar 0.8 mm); **D** Multiplication of shoots from the inoculated callus pieces in shooting media (bar 10 mm); **E** Elongated shoot in multiplied shoots in shoot elongation media (bar 1.2 cm); **F** Root induction from elongated shoots in root induction media (bar 2.5 cm); **G** Primary hardening of pea regenerants in paper cup of soil mix **H** The secondary hardening of regenerants in big pot soil mix under greenhouse environment.

CONCLUSION

This PGR-assisted indirect organogenesis strategy was used to produce an effective callus dependent tissue culture procedure for the recalcitrant Indian pea cultivar ArkaSampoorne, which positively boosted callus induction, shoot multiplication, and root induction of pea regenerants. This standardised

indirect organogenesis technique for pea (cv. ArkaSamoorne) might be immensely important in maximising the chances of successful tissue culture-dependent gene modification in pea.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ABBREVIATIONS

PGRs	Plant growth regulators
2, 4- D	2, 4- Dichlorophenoxyacetic acid
BA	6-benzyladenine (or) Benzyladenine
NAA	1-Naphthaleneacetic acid
cv.	Cultivar

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