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Research Article
Embryo and Ovary Culture of Important Legume Horse Gram, *Macrotyloma uniflorum* (Lam.) Verdc

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Abstract

Background and Objective: *Macrotyloma uniflorum* is nutritious and a good source of bioactive substances with high biological processes. The high frequency and efficient *in vitro* regeneration protocol have developed first time by utilizing embryo and ovary explants in Horsegram, *Macrotyloma uniflorum*. **Materials and Methods:** This research was carried out from horse gram paiyur-2 variety seeds derived embryo and ovary explants used by MS medium supplemented with cytokinins and auxins. **Results:** The phytohormone 0.4 mg L^{-1} zeatin induced a maximum of 12 and 9 shoots from an embryo and ovary explants, respectively. The maximum frequency of rooting with about 9 roots was procured from the shoots cultured from embryonic explants using 0.2 mg L^{-1} NAA and 7 roots were propagated from shoots derived from ovary explants. Overall studies observed that the embryonic explants show a high percent frequency of shoot and root induction when compared to ovary culture and further plantlets rose using embryonic explants with shoot growth period than the ovary explants. **Conclusion:** *In vitro* legume regeneration utilizing embryo and ovary is efficient, dependable and has fewer problems. Embryo explants were used to induce high-frequency plant regeneration, indicating that the ovaries are useful in genetic transformation.

Key words: Horse gram, embryo, ovary, micropropagation, regeneration

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Macrotyloma uniflorum is a nutritious but underused legume found mostly in Africa, Australia and India's semi-arid regions[1]. It is a good source of protein, starch and other bioactive substances (phenols, flavonoids and lignans) with high biological processes and its intake is frequently linked to a low-glycemic-index diet and weight loss[2]. In India, it is known as the "poor man's pulse" and is used as a staple food[3]. Decades-long, plant breeders have been cultivating embryos and ovaries for a better harvest. Processing legumes cause changes in the chemical and functional characterization of proteins, resulting in enhanced grain functionality[4]. The embryo is the tiny sporophyte that results from the fertilized egg or zygote in angiosperms. Plant growth regulators, both natural and synthetic, were widely applied to crops for induction of drought tolerance[5]. Since then, the embryo culture method has been widely used to develop hybrids that were not possible in plants due to embryo abortion and embryo-related problems. Plant tissue culture and genetic engineering are two novel approaches that have been used in plant development across the world. Rather than traditional plant breeding, these approaches assist reduce the time it takes to create plants with desirable characteristics[6]. Plant tissue culture techniques have been widely utilized for clone micropropagation following breeding, particularly in screening for stress tolerance in plants such as salt tolerance and cold tolerance[7]. When mature embryos are used as explant sources, the frequency of plant regeneration is low as compared to immature embryo culture[8]. Furthermore, mature embryos were assisted directly from seeds by non-endosperm[9-11], Slim bits of ripe embryos[12] and mature embryos supported by endosperm[13,14] have been used for callus formation and plant regeneration.

In vitro tissue culture has shown to be a valuable technique for improving these species' genetics, mass propagation and transformation[15]. Using the integrated *in vitro* pollination and fertilization protocol, ovary culture was used to test media effects on the growth of ovules. It was not possible to raise the total number of hybrid plantlets in a single interspecific cross with successful interspecific combinations. Stages of ovary development, in most cases, the optimum stage for ovary culture is the nearly mature embryo sac. This efficient method is extensively used in plant breeding[16].

Plant tissue culture is a technique for growing plant cells, tissues and organs in a controlled environment *in vitro*[17], it's also commonly utilized as a technique for bulk multiplication of medicinal crops[18,19]. Despite the remarkable advantages of tissue culture for clonal and macro propagation, few have been applied commercially. Most previous studies failed to maintain plants derived from tissue culture due to their low environmental adaptability and susceptibility to contamination[20,21]. The use of immature embryos as the explant is labour-intensive as plants must be maintained and grown almost to maturity to obtain the young embryos. There are studies on the production of embryogenic callus from the explants of horse gram[22] and immature cotyledons[23]. But the usage embryos collected from mature *in vitro* germinated seeds and ovaries were collected from *in vivo* sterilized flowers. Embryo and ovary explants have not yet been reported in horse gram. Hence, the present study was framed to select species that have a greater response, induction rates, optimal conditions and development stages of shoots and roots from the embryo and ovary of the genus.

MATERIALS AND METHODS

Study area: This study was carried out from February to August, 2019.

Seeds collection and sterilization: The horse gram seeds variety (Paiyur-2) was procured from Tamil Nadu Agricultural University (TNAU), Regional Research Station, Dharmapuri district. The *in vivo* seeds and flowers were initially rinsed with running tap water for 15 min and then 3% sodium hypochlorite for 5 min, followed by 70% ethanol washes for 2 min. Further, the seeds were washed thrice in sterile double-distilled water and the surface sterilization was continued with 0.1% mercuric chloride (w/v) for 3 min. Finally, the seeds were washed thrice using sterile double distilled water. The surface-sterilized seeds were inoculated in humidified tissue paper in autoclaved Petri-plates and incubated in the light condition for germination (25±2°C with 50 μM m^{-2} sec^{-1}) at 27°C.

Explants inoculation: The dissected ovaries (0.4 mm) from the 50th day *in vivo* flowers were inoculated vertically in a suitable medium. The dissected embryos (0.7 mm) from *in vitro* germinated seeds were placed horizontally in the medium. Both the ovaries and the embryos were inoculated in the culture tubes consisting of a 10 mL MS basal medium consisting of 3% sucrose (w/v) and plant growth regulators (0.1-0.5 mg L^{-1} of zeatin, 0.1-0.5 mg L^{-1} 2iP and a combination of zeatin and TDZ in the ratio of 0.4:0.1-0.5 mg L^{-1}). Whereas, the MS medium without the plant growth regulators served as the control. The pH was adjusted to 5.8 and 0.8% agar (w/v) was added. The medium was autoclaved at 15 psi pressure for about 20 min at 121°C. Once the medium was solidified, the culture tubes were incubated in 16/8 hrs photoperiod under white-cool fluorescent light (25±2°C with 50 μM m^{-2} sec^{-1} at 5±2°C) and 60-70% humidity.

Development of shoots and roots: The individual elongated shoots from the immature embryo and ovary explants were sub-cultured, respectively on a root induction medium comprising various concentrations of auxins such as NAA (0.1-0.5 mg L^{-1}), NAA (0.2 mg L^{-1}) and Picloram (0.1-0.5 mg L^{-1}), to optimize the ideal concentration for root induction. Medium containing without any plant growth regulators was maintained as control.

Acclimatization: The regenerated plantlets were rinsed gently under running tap water to remove the culture medium and immediately planted in pots containing a mixture of soil, sand and vermiculite (1:1:1) (v/v). The potted plants were acclimatized and subsequently moved to the greenhouse[24].

Statistical analysis: For each experiment, a minimum of 50 replicates were taken and triplicates were maintained. The data were presented as Mean±SE for all the treatments. The significant difference between the treatment means was analyzed using a One-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at a $p<0.05$ significant level.

RESULTS

Effect of embryo culture on various plant growth regulators: Immature embryos were excised from *in vitro* germinated seeds for direct embryogenesis on MS media containing different concentrations of zeatin, 2iP and TDZ in Fig. 1a. Embryos started to germinate 7 days after culturing in Fig. 1b. The maximum percentage of shoots 88.00±1.52% was observed in the medium supplemented with (0.4 mg L^{-1}) zeatin using embryonic explants in Fig. 1c. The maximum numbers of 12.66±0.33 shoots/explant were obtained with MS media containing (0.4 mg L^{-1}) zeatin after 8 days of initial culture in Fig. 1d-f. The explants cultured in the control medium (without plant growth regulator) responded poorly and showed 23.00±0.33% shoots from embryo explants. Consequently, all regenerated shoots were morphologically similar in different hormonal combinations tested. So, it is pointed out that media enriched with a high level of zeatin can be used to enhance regeneration and transformation of horse gram using mature embryo explants.

Effect of ovary culture on various plant growth regulators: For *in vitro* ovary culture, healthy ovaries (0.4 mm diameter) were dissected from the 50th day *in vivo* flower in Fig. 2a. As none of the basal media was supported for further sustained growth of shoot buds from ovary explants, therefore, several combinations of different cytokinins were supplemented to the MS basal medium. The further responses of ovary-derived shoot buds to these combinations are summarized in Table 1. The original colour of the explants was changed into darkish green within a few days of inoculation in Fig. 2b. The multiple shoots initiate after 11 days of inoculation in Fig. 2c. Among different combinations tested, ovary explants with supplementation (0.3 mg L^{-1}) showed the highest response of 73.75±3.75% with the number of shoots 9.00±0.40 responded at the end of 11th day from the initiation of culture in Fig. 2d and e. On the other hand, the addition of the zeatin with TDZ combination resulted in a reduced number of shoots and an increased height of the shoots. The MS medium amended with zeatin (0.4 mg L^{-1}) with TDZ (0.2 mg L^{-1}) showed the best result for the length of shoots 8.80±0.24 cm in Fig. 2f and g. The explants cultured in the control medium (without plant growth regulator) responded 19.00±2.48% in ovary explants with the number of shoots 2.88±0.47, respectively during the 13 days of the culture period followed by elongated shoots were excised and transferred into the root induction medium.

Effect of auxins for rooting induction: The rooting response, as well as the nature of roots varied with the different concentrations of auxins, was used. Among different PGR tested, NAA was found to show the best response in rooting in Table 2. The maximum frequency of 89.00±1.15% of rooting was observed in the shoots derived from embryonic explants for 9.66±0.33 number of roots with a length of 9.80±0.11 cm when NAA (0.2 mg L^{-1}) was used in root induction medium and initiated at the 4th day after exposure in Fig. 1g and h. The shoots cultured from the ovary explants produced the rooting frequency of 78.00±0.40% with 8.00±0.00 number of roots and 7.82±0.04 cm length was observed on the 7th day of culture was obtained with combinations of NAA (0.2 mg L^{-1}) with Pic (0.4 mg L^{-1}) in Fig. 2h. The shoots derived from embryonic culture exposed to root induction medium without any PGR resulted in poor response as 39.33±5.20% and induced a lesser number of roots 2.33±0.33 with an average length of 3.33±0.39 cm within 9 days. The shoots derived from ovary explants produced 32.75±0.94% of roots with 2.25±1.45 number of roots and a length of 2.90±0.14 cm within 13 days without any PGRs (Plant Growth Regulators).

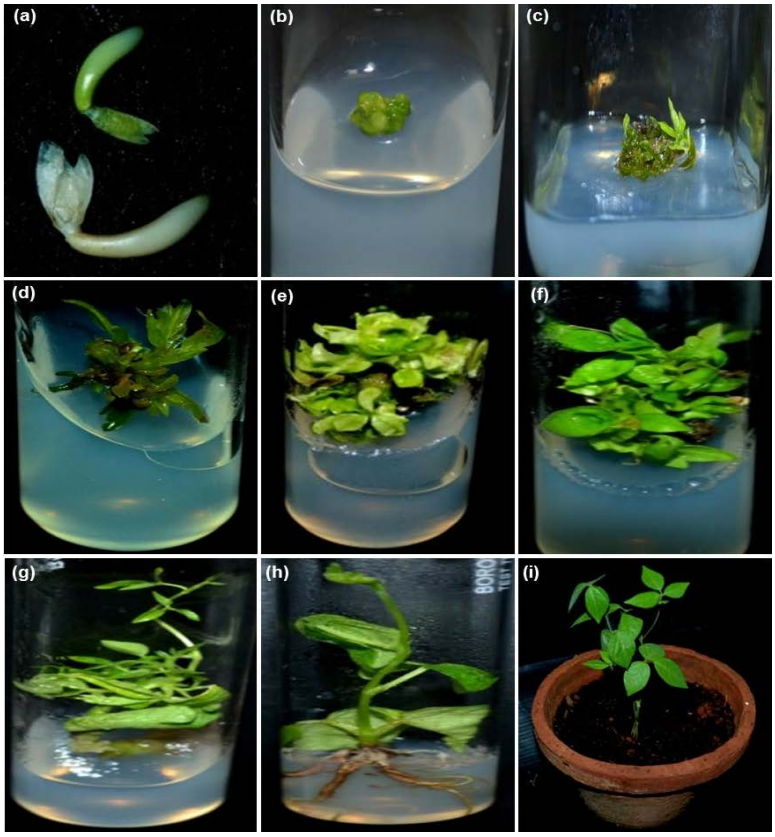

Fig. 1(a-i): Illustration of the main steps of *Macrotyloma uniflorum* regeneration from an immature embryo, (a) Embryo explant, (b) Embryogenic axis developed, (c) Initiation of multiple shoots in the MS medium supplemented with zeatin (0.4 mg L^{-1}), (d-f) Proliferation of shoots and (g-h) Initiation of roots and elongated rooted shoots in the root induction medium with NAA (0.2 mg L^{-1}) and (i) Hardened plants

Table 1: Effect of cytokinins on multiple shoot induction from an embryo and ovary explants of Horse gram, *Macrotyloma uniflorum*

Explants	Embryo				Ovary			
Plant growth regulators (mg L^{-1})	Initiation of days	Explant responding (%)	Number of shoots	Shoot length (cm)	Initiation of days	Explant responding (%)	Number of shoots	Shoot length (cm)
Control	11	23.00±0.33^{j}	3.33±0.33^{j}	3.50±0.28^{i}	13	19.00±2.48^{g}	2.88±0.47^{gh}	1.90±0.40^{i}
ZEATIN								
0.1	10	69.33±0.66^{gh}	5.67±0.57^{i}	5.26±0.14^{fg}	8	37.00±4.33^{f}	6.00±0.40^{efg}	4.50±0.28^{h}
0.2	10	73.00±1.00^{ef}	8.00±0.57^{fg}	8.26±0.14^{c}	9	73.50±1.32^{a}	7.50±0.28^{bc}	7.00±0.00^{cd}
0.3	8	75.00±1.15^{de}	10.00±0.00^{cd}	6.67±0.24^{d}	9	58.50±1.93^{bc}	6.12±0.42^{efg}	6.25±0.25^{ef}
0.4	8	88.00±1.52^{a}	12.66±0.33^{a}	10.00±0.00^{a}	8	60.25±3.68^{bc}	5.50±0.64^{fgh}	5.25±0.25^{g}
0.5	8	79.33±0.88^{bc}	7.33±0.16^{b}	5.96±0.88^{e}	9	48.50±3.27^{de}	4.25±0.25^{gh}	3.60±0.19^{i}
ZEATIN+TDZ								
0.4+0.1	7	71.66±0.88^{efg}	7.33±0.20^{g}	4.16±0.17^{h}	8	40.00±2.04^{ef}	4.00±0.40^{efg}	7.27±0.16^{c}
0.4+0.2	8	76.66±0.22^{cd}	8.42±0.31^{efg}	6.53±0.14^{ef}	8	64.00±2.27^{b}	6.25±0.75^{bcd}	8.80±0.24^{a}
0.4+0.3	8	81.00±0.57^{b}	9.33±0.20^{cde}	7.33±0.14^{c}	8	45.50±2.21^{def}	5.75±0.47^{fg}	7.97±0.07^{b}
0.4+0.4	8	67.33±0.33^{h}	6.00±0.57^{i}	6.76±0.06^{d}	9	41.50±4.64^{ef}	4.75±0.47^{fgh}	6.20±0.31^{ef}
0.4+0.5	10	63.00±1.15^{i}	5.86±0.33^{j}	4.90±0.15^{g}	9	37.25±4.32^{f}	3.50±0.28^{h}	5.20±0.10^{g}
2ip								
0.1	11	70.33±0.22^{i}	8.66±0.33^{j}	6.56±0.14^{bc}	9	53.25±3.03^{cd}	4.00±0.47^{fg}	3.60±0.19^{i}
0.2	9	76.66±0.33^{bcd}	10.53±0.33^{bc}	8.72±0.57^{ab}	9	59.75±4.06^{bc}	6.75±0.25^{ab}	5.07±0.33^{gh}
0.3	9	74.66±1.20^{de}	6.33±0.66^{ij}	7.23±0.18^{d}	11	73.75±3.75^{a}	9.00±0.40^{a}	7.50±0.10^{bc}
0.4	11	71.33±0.88^{fg}	9.00±0.57^{def}	5.86±0.66^{e}	11	68.50±1.19^{ab}	7.75±0.47^{ab}	6.57±0.28^{de}
0.5	7	67.33±1.20^{h}	7.33±0.33^{g}	4.76±0.25^{d}	11	63.75±2.89^{b}	5.25±0.25^{cde}	5.72±0.28^{fg}

The explants treated without plant growth regulators are considered as a control. For each treatment, 50 explants were used and the experiment was repeated three times. Values represent the Means±Standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's Multiple Range Tests at the 5% level

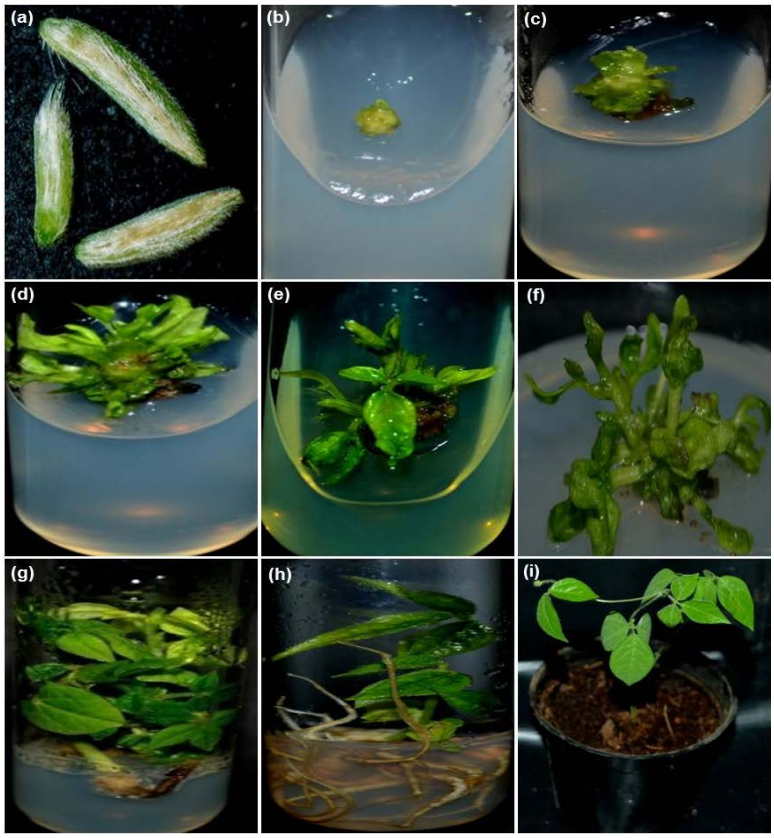

Fig. 2(a-i): *In vitro* culture of ovary and regeneration of plantlets in horse gram, *Macrotyloma uniflorum*, (a) Ovary explant, (b) Developmental stage of ovary, (c) Initiation of multiple shoots in the MS medium supplemented with 2ip (0.3 mg L^{-1}), (d) A regenerated shoots with a terminal bud from ovary explants, (e-f) Proliferation of shoots, (g-h) Rooted elongated shoots in the MS medium supplemented with NAA (0.2 mg L^{-1}) and (i) Hardened plant

Table 2: Effect of auxins on rooting of elongated shoots cultured from embryo and ovary explants of Horse gram, *Macrotyloma uniflorum*

Explants	Embryo				Ovary			
Plant growth Regulators (mg L^{-1})	Initiation of days	Roots responding (%)	Number of roots	Root length (cm)	Initiation of days	Roots responding (%)	Number of roots	Root length (cm)
Control	9	39.33±5.20^{l}	2.33±0.33^{i}	3.33±0.39^{m}	12	32.75±0.94^{i}	2.25±1.45^{l}	2.90±0.14^{l}
NAA								
0.1	6	83.00±1.15^{b}	5.00±0.57^{cd}	7.56±0.08^{cde}	7	65.50±0.64^{h}	3.00±3.00^{kl}	3.72±0.15^{k}
0.2	4	89.00±1.15^{a}	9.66±0.33^{a}	9.80±0.11^{a}	7	68.00±0.00^{g}	4.00±0.40^{fij}	4.45±0.36^{j}
0.3	4	81.66±1.20^{bc}	7.66±0.33^{ab}	8.60±0.15^{bc}	9	71.50±0.28^{ef}	6.00±0.00^{bc}	5.62±0.75^{e}
0.4	6	77.00±1.00^{cde}	5.00±0.57^{ef}	6.40±0.15^{efi}	9	75.00±0.40^{bc}	5.00±0.40^{de}	6.22±0.47^{cd}
0.5	6	73.00±1.52^{efi}	4.00±0.00^{ef}	4.56±0.12^{kl}	9	69.00±0.40^{de}	3.25±0.50^{jk}	5.00±0.61^{fi}
NAA-Pic								
0.2+0.1	6	63.33±0.88^{k}	4.00±0.00^{ef}	3.93±0.17^{lm}	9	68.00±0.00^{fg}	3.50±0.28^{ijk}	4.45±0.13^{j}
0.2+0.2	6	67.33±0.88^{ij}	5.00±0.57^{ef}	5.16±0.17^{jk}	7	71.50±0.50^{def}	5.25±0.25^{cd}	5.22±0.04^{f}
0.2+0.3	8	74.00±1.00^{cde}	6.00±0.00^{cd}	7.20±0.15^{def}	7	75.25±0.25^{ab}	6.25±0.47^{ab}	5.59±0.06^{de}
0.2+0.4	8	70.33±0.88^{fi}	7.00±0.57^{bc}	8.33±0.39^{bcd}	7	78.00±0.40^{a}	8.00±0.00^{a}	7.82±0.04^{a}
0.2+0.5	8	78.33±0.88^{bcd}	8.66±0.33^{a}	6.23±0.14^{fij}	7	74.25±0.25^{cd}	4.50±0.28^{def}	6.55±0.12^{bc}
BAP								
0.5	8	60.33±1.20^{k}	3.33±0.33^{f}	5.83±0.44^{ij}	8	71.25±0.25^{cd}	3.50±0.57^{ijk}	4.55±0.16^{j}
1.0	7	64.66±0.88^{jk}	5.66±1.20^{cd}	6.46±0.84^{efi}	9	73.25±0.25^{ab}	4.25±0.25^{efg}	4.67±0.12^{ij}
1.5	7	71.66±0.88^{fi}	6.00±0.00^{cd}	7.26±0.26^{def}	9	70.00±0.00^{bc}	5.00±0.00^{de}	5.77±0.09^{e}
2.0	5	75.00±0.57^{def}	7.66±0.33^{ab}	9.36±0.87^{ab}	8	67.00±0.70^{fg}	6.00±0.00^{bc}	6.80±0.04^{b}
2.5	7	72.66±0.33^{efi}	5.00±0.57^{ef}	4.60±0.36^{kl}	8	62.75±0.25^{fg}	3.25±0.25^{jk}	3.05±0.05^{l}

The explants treated without any plant growth regulators are considered as a control. For each treatment, 50 elongated shoots were used and the experiment was repeated three times. Values represent the Means±Standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's Multiple Range Test at the 5% level

Acclimatization observation: The well-rooted plantlets were transferred to plastic cups containing sterile sand, soil and vermiculite (1:1:1 v/v/v) and covered to ensure high relative humidity. The plantlets were maintained under controlled environmental conditions for 2 weeks and were irrigated during alternated days to avoid desiccation. The survived plantlets 81% (in embryo explants) in Fig. 1i and 70% (in ovary explants) were subsequently transferred to the earthen pots in Fig. 2i.

DISCUSSION

Earlier works have been able to induce the horse gram plant regeneration and organogenesis of plantlets using shoot tip[25], leaf[22], immature cotyledon[23], cotyledonary nodal[26] explants and recently[27] done the embryo culture. But no attempt was made to culture the embryo and ovary explants. In this study, an efficient embryo and ovary culture protocol has been obtained to recover *M. uniflorum* embryo and ovary explants. To increase the number of viable embryos and ovaries via *in vitro* techniques, future work should entail various hormones and concentrations of the different embryo and ovary ages. Among other varieties of horse gram paiyur-2 variety exhibited a maximum germination percentage of 93%[26]. So, this study was carried out in the paiyur-2 variety.

In the case where long dormancy triggers breeding cycle extension, embryo culture is also helpful in reducing the breeding cycle of new varieties. The present work indicates the regenerative potential of embryos. The embryo axes were successfully used[28,29]. In the present study, the size of the embryo explant is 0.7 mm whereas, the size of the ovary explants is 0.4 mm. Embryo culture is simpler than ovary culture because the dissection of an embryo can be done more efficiently than of the ovary.

Cytokinins are essential for the stimulation of cell division in plants[30]. The shoot inductions were achieved by 88 and 73% from an embryo and ovary explants supplementation with 0.4 and 0.3 mg L^{-1} zeatin, respectively (Table 1). A previous study by Mitrofanova *et al.*[31] showed supplementation of 0.0018 mg L^{-1} of zeatin initiated callus development and gradual increase of zeatin level up to 0.5 mg L^{-1} obtained well-developed shoots[32]. Further study demonstrated that explants exposed to a medium containing 3.0 mg L^{-1} zeatin achieved 50% shoot responses and about 4.2 cm average shoot length[33]. In addition to that, 5 mg L^{-1} kinetin combined with 1 mg L^{-1} NAA induced 71% shoot proliferation[34,35].

In the present study, elongated shoots were exposed to the medium without any plant growth regulators induced 2 roots/shoot. In contrast, a previous study showed none of the roots was produced when explants were exposed to a medium without any PGRs[36]. In this study, 75% of root proliferation was induced by exposing elongated shoots on a medium containing 2.0 mg L^{-1} IBA, in which 7 roots/explant with an average length of 9.36 cm was achieved by using embryonic axis explants. But, a previous study showed that only 13% root induction was observed in the medium supplemented with 0.0001 mg L^{-1} IBA and further 100% root induction was achieved when the explants were exposed to a medium with the combination of 1 mg L^{-1} IBA and 0.5 mg L^{-1} IAA were used[25]. In other medicinal plants, the effect of IBA on root induction and proliferation has been achieved[37,38]. While the shoots of the ovary explants exposed to the rooting medium produced 67% of root induction with 6 roots/shoot and an average 6.80 cm root length. Also, study reported that exposing explants in a rooting medium supplemented with 2500 ppm level of IBA induced 73.3% root response with 8.2 roots/explant and an average root length of 11.8 cm of *in vivo* studies in *Euphorbia tirucalli*[39].

In the present study 81 and 70% of plantlets were successfully acclimatized and established in the 1:1:1 ratio of sand, soil and vermiculite. In a previous study, only 5% of the plantlets survived when transferred to the pot containing sterile soil[40]. Whereas, Tejavathi *et al.*[25] obtained 60% of plant's survival using soil, sand and manure in the ratio of 1:1:1. In contrast, Cenkci *et al.*[41] showed 86% of plants survived in the pot with a mixture of soil, perlite and sand in the ratio of 1:1:1.

CONCLUSION

The explant's physiological state greatly affects the initiation process of *in vitro* regeneration. The highest regenerable potential is recovered in the embryo and ovary explants. Based on the results, it was concluded that *in vitro* legume regeneration using embryo and ovary culture is efficient with fewer difficulties and is highly reliable. High-frequency plant regeneration was achieved using embryo explant than the ovary explant which could be helpful in the mass multiplication of crop production and application of genetic manipulation studies.

SIGNIFICANCE STATEMENT

This study optimized and improved the *in vitro* culture of this important legume crop using embryo and ovary explants that can prevent embryo degradation at the early stage of development shortens the breeding time and allows plants to reproduce efficiently from a single embryo. This prevents seed dormancy from breaking and is also much useful for genetic

transformation studies and to avoid embryo abortion eliminated at an early stage finally, this study will help the researchers to uncover the critical areas of embryo culture with efficient and improved cultivar generation and breeding techniques that many researchers were not able to explore. Thus a new study on embryo and ovary culture by *in vitro* tissue culture method has helped to improve this crop.

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