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IN VITRO APPROACHES FOR SHORTENING GENERATION CYCLES AND FASTER BREEDING OF LOW β-N-OXALYL-L-α, β -DIAMINOPROPIONIC ACID CONTENT OF GRASS PEA (*LATHYRUS SATIVUS* L.)

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ABSTRACT

Grass pea (*Lathyrus sativus* L.) grows as long day plant under natural conditions with single breeding cycle per annum. There is a need to develop strategies by accelerating breeding activities, making it possible to grow more than one generation per annum. This study aimed to search alternative in *vitro* mopho-physiological approaches to induce earlier flowering and accelerate breeding cycles. Nodal junctions with internodes were used as explants to multiple shoots using different concentrations of 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA). The MS medium supplemented with 2 mg L^{-1} BAP and 1 mg L^{-1} NAA was most optimum for production of multiple shoots. These shoots were transferred to MS medium containing 0.1- 0.50 mg L^{-1} BAP singly for generative transformation and growth. The MS medium supplemented with 0.25 mg L^{-1} BAP was most optimum for induced floral buds and flowers within two weeks of culture showing an average number of 4.30± 0.32 flowers per plantlet that continued and increased in number during rooting and acclimatization. The plantlets did not face any problem during acclimatization in the greenhouse, where they set seeds. The maximum β-*N*-oxalyl-l-α, β-diaminopropionic acid (β-ODAP) accumulation was recorded (1.48%) at early developmental stage followed by the flowering stage of grass pea. The results are very encouraging and the methodology used in this experiment could positively contribute towards accelerated breeding of ODAP free grass pea varieties.

KEYWORDS:
Grass pea, *In vitro* flowering, *Lathyrus sativus* L., Nodal junction, ODAP

INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is a highly neglected and orphan crop plant that grows well both under South Asian high-temperature subtropical [1, 4] and Mediterranean temperate West Asian climates [2, 3] with maturity periods of 80-100 and 120 - 140 days respectively [4]. It is not possible to grow 2-3 generations of grass pea unless geographical differences in latitude and altitude are exploited [4, 5] under synthetic (greenhouse) conditions.

Genetic and environmental factors influence grass pea cross pollination with a range of 9.8 to 27.8% [6]. Appreciable progress has not been made in the advancement of grass pea breeding using genetic, genomic, biochemical, molecular, morpho-physiological, approaches in the past [4, 8, 10]. After physiological maturity and anthesis, grass pea takes about 25-45 days (depending on genotypes and environments) to pod filling [5]. If induction of flowering under *in vitro* conditions could be manipulated, it may help in shortening and synchronization of flowering duration events during breeding among varieties and species of the same genus enabling hybridization among them. Complicated crossability relationships in *Lathyrus* have been grouped into primary, secondary and tertiary gene pools [11]. The presence of a high frequency of polyploid cells in *L. latifolius* × *L. heterophyllus hybrid* (WP × het2) is a reflection of numerical chromosomal instability that has also been reported for several Lathyrus species and hybrids [7, 8, 9].

Selection in segregating populations is a very important step in plant breeding. Time taken to obtain a fixed line with desired (e.g. flowering) traits is often a very critical factor in slowing down the genetic gains in breeding programs [13]. It is a complex process regulated by a combination of genetic and environmental factors like light, pH, carbohydrates and plant growth regulators (PGRs) used in the culture medium [8, 14, 16, 17]. *In vitro* flowering has been reported in many species like *Pisum sativum* [8], *Oscimum basilicum* [18], *Phoenix dactylifera* [19] and roses [20] etc. All previous studies show distinct visible gaps in current under-

standing of flowering mechanisms [22, 26, 27]. The breeders are continuously in search of new methods that could accelerate the process of conversion of vegetative to generative growth and induce precocious flowering.

However, grass pea use in food is very limited due to presence of antinutritional components ß-N-Oxalyl-L- α, ß-diaminopropionic acid (ODAP) that is responsible for neuro-lathyrism of lower limbs in humans and animals [4,]. A potential β-ODAP (β-*N*-oxalyl-l-α, β-diaminopropionic acid) free genotype has been identified in *L. tingitanus*, that can be used to develop toxin-free grass pea varieties [7] by backcross. However, such transfer through traditional crossing is restricted among readily crossable species like *L. cicera* and *L. amphicarpus* [12]. It is very difficult to understand flowering behavior of plants under natural conditions that has major effect on plant yield, productivity [21, 22] and breeding activities. Biotechnological approaches could prove an excellent tool to transfer genes from secondary and tertiary gene pools. Therefore, development of genetic resources is of paramount importance for grass pea breeding [4, 5, 9]. Tissue culture techniques have potential to significantly shorten growth cycle and establish cultures that can be used in breeding [8, 13, 14] and genetic transformation studies [15]. Establishing a reliable *in vitro* protocol to induce precocious or adjusted time flowering in grass pea could be an excellent and convenient tool to switch vegetative growth to reproductive growth and flowering for utilization in breeding programs.

There is no report showing genetic information regulating transition from vegetative to flowering stage in most of the plants including grass pea [25]. There are a few reports on in vitro regeneration of shoots and roots in grass pea [12, 23, 24], and any study relating to induction of flowering under *in vitro* conditions has not been reported yet. Therefore, the present study aimed to design a morpho physiological approach for mid to late flowering grass pea cv. Gurbuz to improve *in vitro* shoot regeneration from nodal junctions as explants and induce early flowering along with carrying out ODAP analysis under controlled photoperiod conditions as a first step towards this direction.

MATERIALS AND METHODS

Seed sterilization. The seeds of grass pea cv. Gurbuz were obtained from the Department of Field Crops, Ankara University, Ankara, Turkey. They were washed in running tap water followed by surface sterilization using 30% H_2O_2 and three drops of Tween-20 for 20 min. This was followed by 3 × 5 min rinsing with sterile distilled water and blotting onto sterilized tissue papers. The sterilized seeds were germinated on paper bridges over sterilized water culture in glass test tubes. The nodal junction (about 0.5 cm portion of internode on both sides) explants were excised aseptically (Fig 1a) from 5 - 6 cm long one week old *in vitro* grown plantlets and cultured on respective treatments of MS regeneration medium.

All culture medium treatments were autoclaved for 20 min at 1.4 kg cm^{-2}, after adjusting their pH to 5.6 - 5.8 with 1N NaOH or 1N HCl. All cultures were grown at 25 ± 2°C with a 16/8-h (light/dark) photoperiod. The light was supplied at photosynthetic photon flux density of 25 µmol m^{-2} s^{-1} using cool-white fluorescent lamps (54 w - T 5 Philips).

Multiple shoots induction and *in vitro* flowering. The nodal junctions were used as explants for organogenesis using MS medium [28] manipulated with 5 concentrations of BAP (0.1, 0.25, 0.50, 1 and 2 mg L^{-1}) + 2 concentrations of NAA (1 and 2 mg L^{-1}) making a total of 10 combinations supplemented with 3% (w/v) sucrose and solidified with 0.3% (w/v) gelrite for shoot multiplication and replicated thrice. The observations were recorded on the shoot induction (%), number of shoots/ explants, shoot length (cm) and callus induction (%) at the end of three weeks.

The multiplied (3 - 4 cm long) shoots were elongated on MS medium containing 0.1-0.50 mg L-1 BAP for three weeks. The observations were recorded for mean shoots length (cm), flowering percentage (%), and number of flowers per shoot at the end of two weeks to induce in vitro flowering and shoot elongation.

Rooting and acclimatization. Elongated (6 - 7 cm long) shoots were used for the rooting on ½ × MS medium manipulated with 0.10, 0.25 and 0.50 mg L^{-1} IBA. All rooting treatments contained 24 elongated shoots divided equally into 8 shoots per replication and each treatment was replicated thrice. Additionally, the shoots were also planted on half strength MS medium as control treatment. The data on root induction (%), number of roots and roots length were recorded after three weeks of culture inoculation.

Healthy (150) plantlets with well-developed roots and generative shoots were selected for acclimatization. They were removed from the root induction medium, washed in running tap water to remove sticking gelrite to the roots followed by transfer to 12 cm diameter plastic pots containing 0.62 liter of either peat moss or peat moss + clay soil (2:1) ratio. Each pot was covered with a transparent polyethylene bag to create a high relative humidity; afterwards, the transparent polyethylene bags were carefully and gradually opened. The acclimatized plants were maintained under ambient daylight conditions at 21±2°C in the laboratory greenhouse.

Determination of ODAP during *in vitro* explants growth. The plant samples were collected on germination (1st week), vegetative stage (3rd weeks), flowering stage (5th weeks), podding stage (8th weeks) and maturity stage (11th weeks) of culture explants. The ODAP analysis during *in vitro* plant growth may be helpful to understand the biosynthesis pattern of ODAP in grass pea. All the plant parts excluding matured seeds were dried at 40 °C for 72 hours and carefully mixed to give reasonable representative samples for grinding. Concentration of β-ODAP in the plant parts and the seeds were measured spectrophotometrically using o-phtalaldehyde method by Rao [29] as modified by Briggs et al. [30]. The modification involved the two times extraction of 0.5 g of the flour with 60% ethanol followed by hydrolysis of the flour with 3M KOH in boiling water bath for 30 min. After centrifugation for 15 minutes, an aliquot (250 µl) of the hydrolysate was diluted with 750 µl water and reacted with 2 ml o-phtalaldehyde reagent. The mixture was incubated at 40°C for 2 hours before measuring the absorbance at 425 nm. The β-ODAP standard curve was calibrated using DAP-HCl (Sigma).

Statistical analysis. The data were subjected to one-way analysis of variance (ANOVA, IBM® SPSS® statistics 24.0 for Windows), and the post hoc tests were performed using either Tukey's b or Least Significant Difference (LSD) test. The treatments were arranged in a completely randomized design. The data given in percentages were subjected to arcsine (√X) transformation [31] before carrying out statistical analyses.

RESULTS

Multiple shoots induction. The seeds showed germination under in *vitro* conditions after two days of culture on paper bridges water culture. The seedlings showed elongation to 5 - 6 cm and development of 4-5 nodes in one-week time. These, nodal junction explants showed significantly different behavior on MS medium containing 10 different BAP+NAA combinations to induce multiple shoots in terms of frequency (%) of shoot induction ($F=23.78$; df =9, 20; $p\leq 0.001$), number of shoots per explant ($F=313.08$; $p\leq 0.001$), shoot length ($F=40.757$; $p\leq 0.001$) and callus ($F=22.05$ $p\leq 0.001$) induction. Shoot regeneration was achieved on all treatments (Table 1). It was observed that the increasing BAP concentrations combination with either 1 or 2 mg/L NAA induced increased shoot elongation.

The results showed no or poor shoot induction and profuse rooting on MS medium containing 0.1 mg L^{-1} BAP plus 1 or 2 mg L^{-1} NAA (Fig 1b). Callus induction was noted on eight and shoot induction was noted on all (ten) BAP-NAA treatments (Fig 1c). The most of explants increased in length, when sub-cultured after three weeks' interval. The maximum number of 15.43 ± 0.02 shoots per explant were also the longest shoots (4.63± 0.93 cm) that were induced on MS medium containing 2 mg L^{-1} BAP - 1 mg L^{-1} NAA (Table 1 and Fig 1d). The results showed that BAP-NAA combinations in the MS medium affected shoot length as well as number of shoots. Reduced number of shoots and shoot length were noted on explants that induced callus as they had shorter nodes that affected the cumulative length of the shoots.

***In vitro* flowering.** The shoots induced on MS medium containing (2 mg L^{-1} BAP - 1 mg L^{-1} NAA) were subcultured to MS medium containing 0.10 - 0.50 mg L^{-1} BAP for in vitro flowering and elongation (Table 2). This induced morphogenetic differentiation from vegetative to regenerative phase after two weeks of culture and induced flowering. The ANOVA results confirmed significant differences in flowering percent ($F=36.18$; df =2, 6; $p\leq 0.001$) and number of flowers per plantlet ($F=14.77$; $p\leq 0.01$).

These shoots had flowering percentage of 39.23 - 90.12 %. Maximum flowering percentage (90.12%) and shoots length (7.88 cm) was obtained on MS medium containing 3% sucrose and 0.25 mg L-1 BAP. No generative phase (flowering) was noted on MS medium (control treatment). The multiplied shoots induced 0.33 - 4.30 flowers per shoot before transferring them to rooting medium (Table 2 and Fig 1e, 1f).

Rooting and acclimatization. The maximum flowering shoots (6-7 cm long) recovered on MS medium containing 0.25 mg L^{-1} BAP were transferred to ½ × MS medium containing 0.10 -0.50 mg L^{-1} IBA for rooting. Root initiation was observed on the shoots 15-16 d after transfer to rooting medium. *In vitro* regenerated flowering plants induced multiple roots at the lower part of the plant. The results showed that rooting percentage and mean root length decreased drastically with increasing concentration of IBA. The rooting percentage and mean number of roots/explant and root length were ranged 14.12 - 83.43 %, 2.96 - 7.33 and 1.66 - 5.63 cm respectively (Table 3). Maximum and longest rooting were induced on 0.1 mg L^{-1} IBA. This flowering or generative stimulus continue flowering during rooting and acclimatization in the greenhouse. Fastest achievement of 100% flowering in two weeks' time was noted on 0.25 mg L^{-1} BAP induced plantlets. While, the plantlets induced on other treatments achieved 100% flowering in 4 - 5 weeks' time in the greenhouse (data not shown). No visible differences were recorded in morphology of flowers obtained on *in vitro* regenerated plants and the plants obtained from seeds. Other combinations

of BAP had negative effect to induce generative shoots with inflorescence.

As explained in materials and methods 150 plantlets transferred to 0.62 liters plastic pots containing peat moss or peat moss-clay soil (2:1) for acclimatization. The peat moss showed development of strong root system and had 0.1 to 0.15 cm thick roots tapered to hair-like structures at growing ends and root nodes by developing filamentous roots with lateral spread. No obstacle was recorded on hardening of the plants.

TABLE 1

Shoot induction from nodal junction explant of grass pea inoculated on MS medium containing BAP (0.10 – 2.0 mg L^{-1}) and NAA (1- 2 mg L^{-1}) after three weeks of culture

MS + Treatments mg L^{-1}		Shoot induction (%)	Number of shoots/ explants	Shoot length (cm)	Callus induction (%)	Callus formation
BAP	NAA					
0.10	1.0	4.73± 0.29 e	0.33±0.03 d	0.13± 0.04d	52.36±2.84 a b	+
0.10	2.0	9.56 ±1.79e	0.33 ±0.01d	0.10 ±0.00d	49.96±3.27 a b	+
0.25	1.0	40.73± 1.15c d	1.30 ±0.07d	0.46 ±0.03c d	37.16± 2.92b c	+
0.25	2.0	20.53±1.06 c d e	1.00± 0.05d	0.53 ±0.08c d	24.00± 2.25c d	+
0.50	1.0	14.23± d e	0.66 ±0.05d	0.43 ±0.03c d	74.40±3.92 a	+
0.50	2.0	46.43 ±1.39b c	1.56 ±0.31d	1.20± 0.05c	31.66± 1.91b c	+
1.0	1.0	71.00± 1.23a b	7.46± 0.16c	2.50± 0.09b	40.60± 2.95b c	+
1.0	2.0	70.86 ±1.59a b	11.96± 0.43b	2.33± 0.80b	0.00±0.00	-
2.0	1.0	87.90 ±1.31a	15.43 ±0.02a	4.63± 0.93a	0.00±0.00	-
2.0	2.0	83.30 ±1.92a	3.60± 0.09d	2.30± 0.06b	66.36± 1.54a	++
MS medium (control)		14.23±1.59de	1.00±0.07d	0.15±0.01d	0.00±0.00	-

Values with in a column followed by different letters are significantly different ($p<0.01$) using Tukey's b test.
*Callus formation: -, Nil: +, Low; ++ Moderate
± Standard error

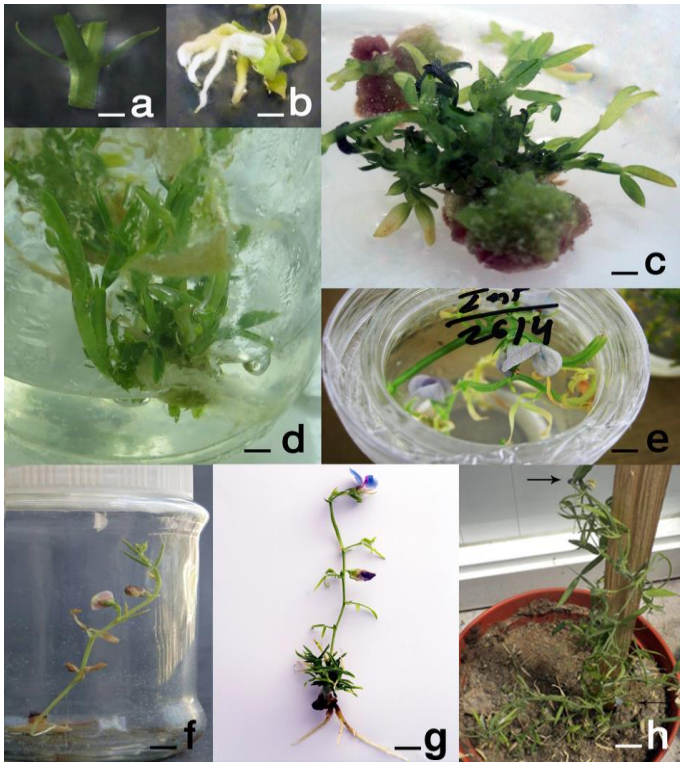

FIGURE 1

Multiple shoots induction and *in vitro* flowering of *L. sativus* cv. Gurbuz:

(a) nodal junction bearing about 0.5 cm portion of internodes on both sides as explants, (b) poor shoot induction and profuse rooting on MS medium containing 0.1 mg L^{-1} BAP and 2 mg L^{-1} NAA, (c) callus induction and shoot induction from nodal junction on MS medium supplemented with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA after 3 weeks of culture, (d) maximum number and longest shoots per explants were induced on MS medium containing 2.00 mg L^{-1} BAP - 1 mg L^{-1} NAA, (e, f) maximum flowering response on MS medium containing 3% sucrose and 0.25 mg L^{-1} BAP after two weeks of culture, (g) rooting using ½ strength MS medium containing 0.1 mg L^{-1} IBA, (h) plant bearing mature pods containing seeds after acclimatization under greenhouse conditions. Bar of Figure 1a, b, c =0.4 cm, Figure 1d, e, f = 0.3 cm, Figure 1g = 2 cm, 1h = 5 cm.

TABLE 2
***In vitro* flowering response of nodal junction explants of grass pea on MS medium containing different concentrations of BAP after two weeks of subculture**

BAP treatments (mg L^{-1})	Flowering percentage (%)	Mean shoots length (cm)	Number of flowers per shoot
0.10	39.23 ±1.15c	5.08± 1.58c	0.33± 0.21b
0.25	90.12 ±3.47a	7.88 ±0.56a	4.30± 0.32a
0.50	62.50± 2.91b	6.86± 1.62ab	0.66 ±0.27b
MS_0 (control)	0.00±0.00	4.00±0.03	0.00±0.00

Values with in a column followed by different letters are significantly different ($p < 0.05$) using LSD test.

TABLE 3
Root induction response of nodal junction explants of *L. sativus* L. on ½ strength MS medium containing different concentrations of IBA

IBA treatment (mg L^{-1})	Root induction (%)	Mean number of roots / explants	Root length (cm)
½ MS medium* (Control)	0.00	0.00	0.00
0.10	83.43± 2.92a	7.33± 0.62a	5.63± 1.46a
0.25	63.30±2.31 b	4.36± 0.33b	3.16± 1.26b
0.50	14.12± 1.09c	2.96± 0.84c	1.66± 0.08c

Values with in a column followed by different letters are significantly different ($p < 0.05$) using LSD test.
* ½ strength of macro, micro elements and vitamins of MS medium

TABLE 4
ODAP analysis of various growth stage of *in vitro* regenerated plant of grass pea

Plant growth stage	Germination (1st week)	Vegetative (3rd week)	Flowering (5th week)	Podding (8th weeks)	Maturity (11th weeks)	Matured Seed
ODAP %± SE	1.48 ± 0.06	0.97 ± 0.05	1.10 ± 0.08	0.84 ± 0.03	0.43± 0.05	0.21 ± 0.03

Plant survival percentage remained 73% on peat moss + clay (2:1) and 89% on peat moss after three weeks of hardening. All the mature regenerated plants induced 4 - 5 pods per plant that developed into normal seeds. All developing pods contained 2 - 3 seeds that were morphologically viable (tetrazolium test) and similar to the seeds obtained from the field grown plants.

ODAP analysis during *in vitro* vegetative, reproductive and maturity stage. During the in vitro explant growth, ODAP concentration changes variably at different plant developmental stages with maximum accumulation ODAP concentration were recorded during early germination stage. The ODAP analysis of *in vitro* regenerated seedlings may helpful to understand the role of micro- and macronutrient content on biosynthetic and how to different environmental and growing conditions affect β-ODAP accumulation in grass pea. The minimum and maximum ODAP content were found in plants at maturity and seed germination stages that ranged 0.21% to 1.48% respectively.

The early developmental (germination) stage showed maximum ODAP accumulation that drastically reduced during vegetative (3rd week) growth that increased again at flowering stage. It may be reasoned that transition from vegetative to generative phase including flowering stage and early developmental stage results in increased accumulation of ODAP. The podding and early maturing stage showed decreasing of ODAP content after flowering (Table 4). The seeds set under *in-vitro* and *ex-vitro* conditions did not show significant difference in ODAP content.

DISCUSSION

Plant regeneration from seeds is very tedious in legumes and grass pea is no exception [32, 33]. Biotechnology tools can facilitate possibility of inducing early generative phase in cultured plants that may help in accelerating breeding by shortening their life cycle [8, 16].

The present study provides a reliable and significantly improved *in vitro* regeneration and accelerated flowering protocol in grass pea compared to previous reports [23, 34]. The present study reports a significant improvement over the results of Barik et al. [23] and Barpete et al. [35]. Barik et al. [23] achieved maximum number of 8.2 shoots per explant with root length of 4.1 cm. whereas; Barpete et al. [35] noted maximum number of 8 shoots per explant with 4 cm long shoots. A comparison of three regeneration studies shows genotype, age and type of explant significantly affects regeneration capacity.

The most important observation in the present experiment pertains to accelerated flowering, never reported or observed earlier in grass pea as the multiplied roots were matured or transformed to generative phase before rooting. The present experimental arrangement helped in accelerated transfor-

mation of *in vitro* regenerated plantlets from vegetative to generative stage bearing fertile floral buds and flowers. Although, Gharyal and Maheshwari [36] reported flower bud formation that could not flowered in control condition in grass pea.

This study reports flowering of grass pea after seven weeks of culture (16-h light photoperiod) under *in vitro* conditions. All flowers matured and set healthy fertile seeds in pods under in vitro conditions. It is assumed that 16 h light photoperiod and regeneration on BAP containing medium stimulated early transition from vegetative to generative growth in line with Heylen and Vendrig [16]. Ammar et al. [19] and Wang et al. [20]; emphasize that *in vitro* floral induction is influenced by thidiazuron (TDZ) or combination of zeatin and NAA. However, auxin is either ineffective [39] or inhibitory [40] for floral development in some species.

The important role of auxin in flower induction and development has been reported in green pea [37] and *Vigna mungo* [38]. Contrarily, this study reports multiplication of shoots followed by their elongation on a medium containing low concentration (0.25 mg L^{-1}) of BAP to make it effective for floral induction. These results are in agreement with Fujioka et al. [41] and Nagdauda et al. [42], who reported *in vitro* flowering in pea and bamboo under the influence of cytokinins. Similarly, Kostenyuk et al. [43] also suggested beneficial effects of cytokinin (2.5 mg L^{-1} TDZ) on the induction of *in vitro* flowering in orchids. Cytokinins induce precocious flowering signals in *Cymbidium niveo* and tobacco as well [44, 45]. First flower induction was noted after one week of transferring the multiplied shoots to medium leading to the development of four to five flowers per plantlet on growing shoots that continued to blossom during rooting and acclimatization under greenhouse conditions. No floral induction was noted on shoots cultured on MS medium without PGR/s. It is assumed that BAP evoked floral induction by early transition from vegetative to generative stage that affected early mitotic activity regulation leading precocious initiation of generative growth [18, 46]. However, grass pea is a long-day plant and flowering is achieved only after 120 - 142 days (17 - 20.9 weeks) on sowing in open fields under conditions reported by Kumar et al. [4]. This is an important development in grass pea and could be used as an excellent tool to accelerate breeding and hybridization by accelerated selection in segregating populations of hybrids and pure lines without dependence on geographical factors or season [8].

Precocious floral induction is meaningless if the shoots cannot be rooted and acclimatized under external conditions. The present study showed distinct effectiveness of ½ × MS basal medium containing 0.10 mg L^{-1} IBA for inducing maximum rooting (83.43± 2.92%), mean number of roots per explants and root length post flowering that the elongated shoots could be rooted without any obstacle on ½ × MS medium containing IBA. Ochatt et al. [14] and Barpete et al. [47] also induced rooting on in vitro multiplied shoots on MS medium containing IBA. Comparing studies by Barik et al. [23] and Barpete et al. [35] with this study, the results emphasize use of low concentration of IBA (0.10 mg L^{-1}) for improved precocious rhizogenesis, number of roots per explant and root length. Barik et al. [23] and Barpete et al. [35] reported 75% and 78% root induction using 0.25 mg L^{-1} IBA or 2.85µM indole-3-acetic acid respectively. The results are not in agreement with Barik et al. [23] in days to achieve rooting; who reported 30 days to root micropropagated plants. Present study reports 7.33 roots per explant with 5.63cm long roots regenerated and matured shoots in 35 days of culture. Barik et al. [23] achieved maximum number of 3.6 roots per explant with root length of 3.9 cm.

Another important aspect of *in vitro* flowering reported in this study is survival of flowering plants outside culture vessels in pots during hardening and acclimatization in the greenhouse. Major problem in most *in vitro* derived plants is a desiccation shock experienced just after transplantation [34, 48]. This is fatal during the initial days of establishment. No negative effects of desiccation shock were observed on the transplanted material in the present study, and most of the transplanted plant recovered in a short time under the greenhouse conditions. Most obvious reason could be the maturing of in vitro regenerated shoots before rooting.

The *in vitro* regenerated grass pea seedling showed ODAP concentration changed during plant growth (Table 4) and maximum ODAP accumulation were recorded in early developmental stage in line with Jiao et al. [49]. They emphasized that accumulation of β-ODAP is higher in young grass pea seedlings probably related to changes in nitrogen metabolism. Similarly, previous studies indicated that β-ODAP content is present in all parts of the plant with the highest amount reported in the leaf and embryo at vegetative and reproductive stages [49, 50, 51, 52]. Moreover, Jiao et al. [49] emphasized that nitrogen supply plays a major role in increasing β-ODAP contents in grass pea.

CONCLUSIONS

This paper presents a simple and reliable morpho physiological approach to achieve an *in vitro* system for increasing number of grass pea generations in a reduced time [(i) one week to obtain explant from germinated seedlings, (ii) three weeks for regeneration, (iii) two weeks for elongation shoots and induction of early flowering, (iv) three weeks for rooting and (v) three weeks for hardening, acclimatization and blooming confirms] of 4.33

generations of grass pea per annum with mid to late flowering genotypes. The morpho physiological observations reported here are novel and this protocol. Grass pea genotypes are usually released after 6 - 8 generations of self-pollination to achieve an appropriate level of homozygosity. Decreasing the length from F_1 to F_7 generation will overcome this breeding bottleneck and accelerate possibility of genetic improvement of grass pea with accelerated selection.

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Surendra Barpete, Priyanka Gupta, Khalid Mahmood Khawar, Sebahattin Özcan, and Shiv Kumar discussed, planned and carried out experiments collectively. SB, PG and KMK made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. SO and SK have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors read and approved the final manuscript to be published.

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