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In vitro plant regeneration via indirect organogenesis from different explants of *Lathyrus sativus* L. and *Lathyrus cicera* L.

Regeneración vegetal *in vitro* vía organogénesis indirecta de explantes diferentes de *Lathyrus sativus* L. y *Lathyrus cicera* L.

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Abstract. The grass pea (*Lathyrus sativus* L.) and flatpod peavine (*Lathyrus cicera* L.) are the most economically important and widely cultivated *Lathyrus* species. However, their utilization is limited due to the presence of their endogenous toxin β-*N*-oxalyl-L-α, β-diaminopropionic acid (β-ODAP). Thus, a β-ODAP free variety should be developed through some plant breeding technique like either mutational breeding or genetic-manipulation. In this circumstance, the plant regeneration of *Lathyrus* species becomes a bottleneck. In the present study, an efficient system for *in vitro* regeneration of *L. sativus* with high β-ODAP levels, and *L. cicera* with low β-ODAP levels, was developed from different explants (axillary buds, leaves and stems). At first, the green nodular calli were induced from sterile seedlings. It was found that the pre-culture of sterile seedlings with 15 mg/L 6-benzyladenine (BA) was necessary for *L. cicera*, but not for *L. sativus*. All of these calli were able to differentiate into adventitious shoot formation when cultured further. Among those explants, leaf segments were the optimum because of their easy obtainment and high regeneration efficiency (i.e., 66.48% in *L. sativus* and 62.13% in *L. cicera*). Furthermore, it was found that the pre-treatment would significantly improve the efficiency for nodular calli induction in both varieties, although it was easier on explants of *L. sativus* than on those of *L. cicera*. When these *in vitro*-derived plantlets of the two *Lathyrus* species were planted on half-strength Murashige and Skoog medium (MS) medium with α-naphthalene acetic acid (NAA) supplemented, 60% of them developed several roots. After being transplanted into soil, above 85% of each *Lathyrus* species grew well. The protocol would be useful for further expanding the propagation and *Agrobacterium*-mediated genetic transformation to obtain low β-ODAP varieties.

Keywords: Flatpod peavine; Grass pea; Green-nodular callus; *In vitro* regeneration.

Resumen. *Lathyrus sativus* L. y *L. cicera* L. son las especies de *Lathyrus* más importantes económicamente y ampliamente cultivadas. Sin embargo, su utilización es limitada debido a la presencia de su toxina endógena β-*N*-oxalic-L-α, β-ácido diaminopropiónico (β-ODAP). De esta manera, se debería desarrollar una variedad libre de β-ODAP a través de alguna técnica de mejoramiento vegetal ya sea cruzamiento por mutación o manipulación genética. En este caso, la regeneración de las especies de *Lathyrus* se convierte en un cuello de botella. En este estudio, se desarrolló un sistema eficiente para la regeneración *in vitro* de *L. sativus* con altos niveles de β-ODAP y de *L. cicera* con bajos niveles de β-ODAP a partir de fuentes diferentes (yemas axilares, hojas y tallos). Primero se indujeron los callos nodulares verdes desde plántulas estériles. El pre-cultivo de plántulas estériles con 15 mg/L de 6-benciladenina (BA) fue necesario para *L. cicera,* pero no para *L. sativus*. Todos estos callos se diferenciaron en la formación de tallos adventicios cuando se continuó con su cultivo. Entre aquellos explantes, los segmentos foliares fueron óptimos debido a la facilidad de su obtención y alta eficiencia de regeneración (66,48% en *L. sativus* y 62,13% en *L. cicera*). Además, el pre-tratamiento mejoró la eficiencia de inducción de callos nodulares en ambas variedades, aunque fue más fácil en explantos de *L. sativus* que en aquellos de *L. cicerea*. Cuando estas plántulas de las dos especies de *Lathyrus* derivadas de cultivo *in vitro* se plantaron en medio Murashige y Skoog de media fuerza con ácido α-naftalenacético suplementado, 60% de ellas desarrollaron varias raíces. Después de ser plantadas en suelo, más de 85% de cada especie de *Lathyrus* creció bien. El protocolo sería útil para seguir expandiendo la propagación y, vía la transformación genética mediada por *Agrobacterium*, obtener variedades con bajos niveles de β-ODAP.

Palabras clave: *Lathyrus sativus; Lathyrus cicera*; Callos nodulares verdes; Regeneración *in vitro*.

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INTRODUCTION

Lathyrus species are important edible and forage crops in those Low Income Food Deficit Countries (LIFDCs) for their cheapest, dietary lysine-rich proteins (Barik et al., 2005a) and adaptation to adverse agricultural conditions such as drought, salinity, low soil fertility, etc (Zambre et al., 2002; Barpete, 2015). Among them, grass pea (*Lathyrus sativus* L.) and flatpod peavine (*Lathyrus cicera* L.) are the most economically important and widely cultivated. Nevertheless, their utilization is limited due to the presence of a neuroexcitatory non-protein amino acid, β-*N*-oxalyl-L-α, β-diaminopropionic acid (β-ODAP) that was considered responsible for human lathyrism (Spencer et al., 1986). Plant breeders have approached to produce some improved varieties with low β-ODAP content (Campbell et al., 1994; Kumar et al., 2011) through conventional plant breeding techniques (Kumar et al., 2013). However, these low-toxin varieties did not show stability on β-ODAP levels at different growing conditions (Jiao et al., 2011a; Barpete et al., 2014a). This is because β-ODAP contents are highly influenced by genotype, external environment factors and their interactions (Jiao et al., 2011a, 2011b; Kumar et al., 2011). Thus, a β-ODAP free variety is yet to be developed. Genetic-manipulation techniques may provide means for achieving free or low β-ODAP varieties in *Lathyrus* (Zambre et al., 2002). Thus, it is imperative to develop an *in vitro* regeneration procedure of *Lathyrus* species (i.e., using a genetic transformation including *Agrobacterium*).

Several studies have attempted to develop an efficient *in vitro* regeneration procedure of *Lathyrus* species (Gharyal & Maheshwari, 1980; Malik et al., 1992; Roy et al., 1991, 1992; Barna & Mehta, 1995; Kunjumon et al., 1996; Zambre et al., 2002; Barik et al., 2005a, 2005b; Kendir et al., 2009; Barpete et al., 2014b). In general, it was developed through an organogenesis pathway (Malik et al., 1992). However, plant regeneration from protoplasts (McCutchan et al., 1999; Durieu & Ochatt, 2000) and somatic embryogenesis (Barna & Mehta, 1995) have also been obtained. It was reported that the i*n vitro* plant regeneration via the organogenesis pathway of *Lathyrus* was developed through apical and axillary buds (Zambre et al., 2002), cotyledonary nodes (Barik et al., 2004), cotyledons (Barik et al., 2005b), embryonic nodes (Barpete et al., 2014c), epicotyls (Barik et al., 2005b), hypocotyls (Barik et al., 2005b), internodes (Roy et al., 1993; Mehta et al., 1994; Barik et al., 2005b), immature zygotic embryos (Kendir et al., 2009), leaves (Mehta et al., 1994; Kunjumon et al., 1996; Barik et al., 2005b), roots (Roy et al., 1992; Mehta et al., 1994), and stem explants (Sinha et al., 1983). Most of the above studies used juvenile tissues containing meristems as explants, but the regeneration procedures were not clearly stated (Zambre et al., 2002).

In this paper, plant regeneration of *L. sativus* and *L. cicera* are reported from green-nodular callus originated from axillary bud, leaf and stem explants. The protocol confirmed the regeneration-competence trait of different explants. It would also be useful for further, expanded propagation via the *Agrobacterium*-mediated genetic improvement as well as for the exploitation of somaclonal variations for low β-ODAP varieties.

MATERIALS AND METHODS

Plant material and culture conditions. In the present study, two *Lathyrus* varieties of grass pea [*Lathyrus sativus* L. cv. LZ(2), with high β-ODAP levels] and flatpod peavine [*Lathyrus cicera* L. cv. LZ(1), with low β-ODAP levels] (Jiao et al., 2006) were used. The seeds were collected from the Key Laboratory of Arid and Grassland Agroecology, Lanzhou University, China.

Seeds of the two varieties were washed thoroughly with running tap water, and then sterilized as described previously (Xu et al., 2009). Afterwards, they were inoculated onto MS medium (Murashige & Skoog, 1962) supplemented with various concentrations of 6-benzyladenine (BA; 0, 1.0, 5.0, 10.0 or 15 mg/L) to obtain germination and pre-culture. One week later, germinating seedlings were used as source of explants.

All the media used in this paper were supplemented with 3% (w/v) sucrose and 0.7% (w/v) Difco bacto agar (Sanland Chemicals, Los Angeles, USA), adjusted to pH 5.8 using NaOH before autoclaving at 121 ˚C for 20 min (1.55 kg/cm^2 pressure). Explants were placed into glass jars (5.0 cm in diameter and 8.0 cm high) containing about 30 mL MS media, and covered with plastic, transparent white screw caps. All cultures were illuminated with cool white fluorescent light of 40 μmol/(m^{2}s) with a photoperiod of 16 h light and 8 h dark at 25 ± 1 ˚C.

Induction of calli and adventitious buds. Axillary bud, leaf, root and stem explants from 7-day-old sterile seedlings with or without pretreatment via BA were placed onto MS medium with different concentrations of 2, 4-Dichlorophenoxyacetic acid (2, 4-D; 0.1, 0.5 and 1.0 mg/L), BA (5.0, 10.0, 15.0 mg/L), or α-naphthalene acetic acid (NAA; 0.1, 0.5 and 1.0 mg/L) to induce callus. About 4 weeks later, the morphological and regeneration-competence trait of calli were observed. Then the calli were transferred onto MS medium with different concentrations of BA (5.0, 10.0, 15.0 mg/L) alone for adventitious bud formation. Each treatment contained at least 60 explants. Visual observations of cultures showing callus induction, shoot differentiation and numbers of shoots per explant were recorded after 30 d.

Rooting and domestication. About 2.0 cm long regenerated shoots were excised and inserted in half-strength MS medium with NAA (0.3, 0.5, 1.0 mg/L) for rooting.

Six weeks later, plantlets with well-developed roots were washed several times with distilled water. Then, they were

transplanted into water-saturated vermiculite and cultured with a 16-h photoperiod at 25 ± 1 °C. To maintain humidity, the pots were initially covered with polyethylene bags, which were removed after 2 weeks.

Statistical analysis. Each treatment had 3 replicates containing at least 10 explants and all experiments were repeated twice. All data were reported as means ± SE. Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., USA). One way ANOVA was performed to evaluate the effectiveness of operation. A $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The regeneration-competence trait of calli. Two *Lathyrus* varieties, *L. sativus* with high β-ODAP levels and *L. cicera* with low β-ODAP levels, were used to study their regeneration capacity. The different explants (axillary bud, leaf, root and stem) from sterile seedlings whether pretreated with BA or not, induced calli under MS medium containing various concentrations of 2,4-D, BA, or NAA (Table 1). These calli showed different color and texture: brown loose, green compact, green nodular, white compact or white loose (Table 1). Among them, green nodular callus induced via the BA treatment could only differentiate into adventitious buds further, whereas, other calli were lacking of regeneration competent in subculture. Generally, the green nodular calli were formed at one (Fig. 1A, 1I) or two (Fig. 1B,1J) cut ends of the stem explants, near the midrib of the leaf surface (Fig. 1C, 1K), at the cut end (Fig. 1D, 1K-M) or near the center of the axillary bud (Fig. 1E, 1N) of the two *Lathyrus* varieties. These green nodular calli were separated, showed compact texture, and were occasionally accompanied with little brown loose or white compact calli.

About one month later, these calli were separated from the original explants and subcultured on MS medium supplemented with various concentrations of BA. Among them, it was only the green nodular calli which could induce into adventitious buds (Fig. 1F, 1G, 1O) after 2-3 weeks, followed by the production of multiple shoots in both varieties. The other calli could proliferate in subsequent cultures, but remained non-organogenic.

In fact, the greenish, brownish, and yellowish callus with loose texture were ever induced from leaf and stem segments of *L. sativus* (Zambre et al. 2002). However, they proliferated in subsequent cultures only, and remained non-organogenic. Therefore, it was thought that the nodular calli induction would be a prerequisite for plant regeneration in *Lathyrus* varieties (Zambre et al., 2002), as we got here. Moreover, nodular calli were also successfully induced from *Beta vulgaris var cicla* (Xu et al., 2009), *Garcinia mangostana* L. (Te-chato & Lim, 2000), *Phaseolus* species (Zambre et al., 2001) and *Pinus radiate* (Schestibratov et al., 2003), which could differentiate into adventitious buds, and further regenerated into plantlets.

Effects of explant types and pretreatment of sterile seedlings on callus induction and shoot formation. It was observed that the explant types affected multiple shoot formation to a great extent in the presence of cytokinin (Xu et al., 2009; Barpete et al., 2014c). Of the different explants tested, the axillary bud, leaf and stem except for root explants could form green nodular calli in both varieties regardless of the pretreatment with BA (Table 2). For axillary bud explants, the green nodular calli formation efficiency ranged from 10.34% to the most 82.30% on *L. sativus*, and 26.78% to 86.59% on *L. cicera* when different BA concentrations were used. At the same circumstance, leaf explants formed the green nodular calli with efficiencies ranging from 5.17% to 66.48% on *L. sativus*, and from zero to 62.13% on *L. cicera*, respectively. The green nodular calli formation efficiency for stem explants ranged from 3.55% to 46.53% on *L. sativus*, and from zero to 50.75% on *L. cicera*, respectively (Table 2). Moreover, the maximum shoot numbers were 10.67 on *L. sativus* and 8.18 on *L. cicera.* We can conclude that the meristem pre-existing explants were more responsive in calli induction and shoot formation than no meristem pre-existing explants in both varieties. This was also reported in most *in vitro* regenerated shoots of *Lathyrus* species (Zambre et al., 2002). Of the different explant types used, leaf explants were the nearest to the optimum because of its high regeneration efficiency *in vitro* and easy obtainment.

The pre-treatment would significantly improve the efficiency for nodular calli induction in both varieties (Table 2). The higher the concentration of BA in pre-culture, the higher the efficiency for obtaining the green nodular calli. However, when

Table 1. Morphologic features of the calli induced via different types of hormones.
Tabla 1. Características morfológicas de yemas axilares, hojas, raíces y tallos inducidos por diferentes tipos de hormonas.

Hormones	Axillary buds	Leaf	Root	Stem
2,4-D	WL, GC	WL, GC	BL, WL	WL
BA	BL, GN	GN, WC ,WL	BL	GN, WC ,WL
NAA	WL, GC	GC, WL	BL, WL	GC, WL

Note: BL- Brown loose, GC- green compact, GN- green nodular, WC-white compact, WL-white loose.

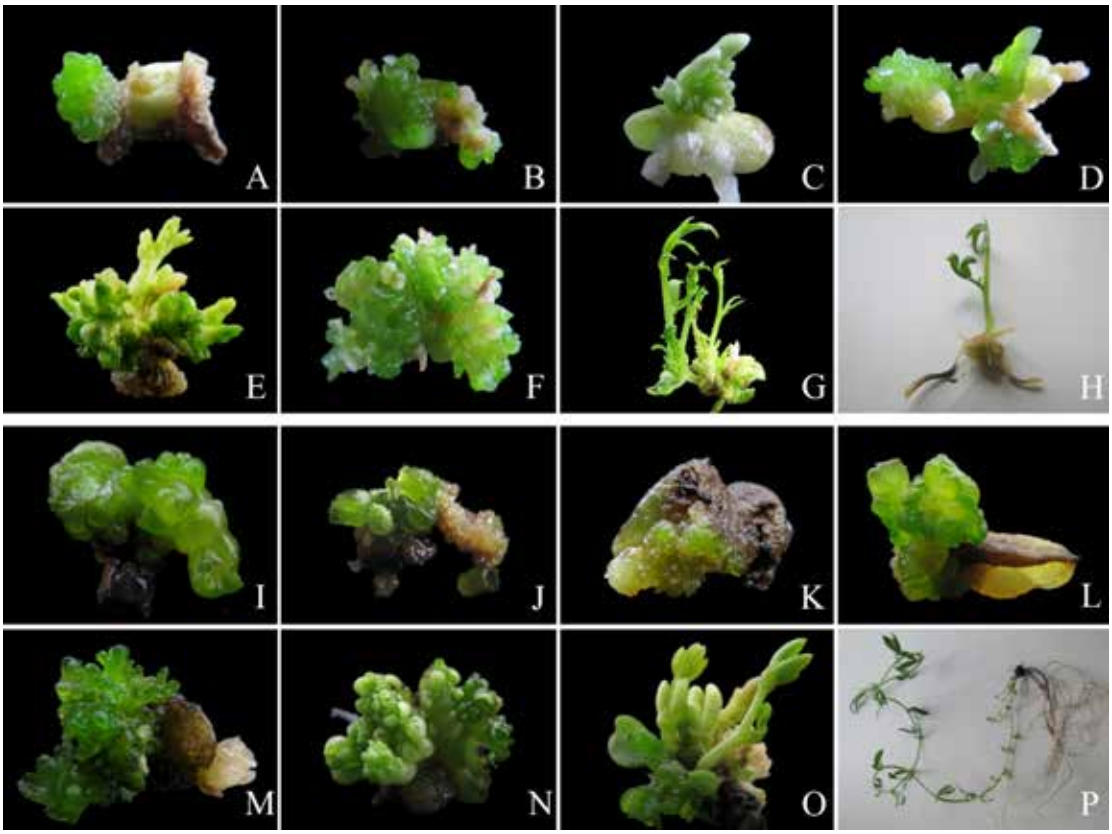

Fig. 1. Plant regeneration of *L. sativus* (A-H) and *L. cicera* (I-P). A-B, I-J: the green nodular calli formed at one or two cut end of the stem explant. C-D, K-M: the green nodular calli formed near the midrib or cut end of the leaf. E, N: calli formed near the center of axillary bud. F-G, O: adventitious buds/shoots. H, P: rooted plantlets.

Fig. 1. Regeneración vegetal de *L. sativus* (A-H) y *L. cicera* (I-P). A-B, I-J: callo nodular verde formado en uno o dos puntos de corte de explantes de tallo. C-D, K-M: callo nodular verde formado cerca de la mitad de la nervadura o corte al final de la hoja. E, N: callo formado cerca del centro de una yema axillar. F-G, O: yemas adventicias/tallos. H, P: parte vegetal regenerada verde con raíces.

the BA concentrations used for pre-culture were higher than the optimum, the efficiency of green nodular calli formation was reduced in leaf and stem explants of *L. sativus* (Table 2). This demonstrates the importance of pre-conditioning of the source tissue on regeneration of multiple shoot buds on several species of *Lathyrus* (Malik et al., 1992; Barik et al., 2004; Barpete et al., 2014c). On the other hand, the threshold level for axillary bud explants of *L. sativus*, and axillary bud, leaf and stem explants of *L. cicera* were not detected. Moreover, it seemed that the pre-culture of sterile seedlings with BA was not necessary on *L. sativus* to induce the green nodular calli with various explants. We observed no shoot regeneration from leaf and stem explants from non-preconditioned *L. cicera*. It suggests that the pre-culture was necessary for *L. cicera* regeneration when leaf and stem explants were used. This difference of the two *Lathyrus* species on the pre-culture of sterile seedlings with BA might relate to their different endogenous hormonal levels (Xu et al., 2009).

It is well known that plant regeneration is strongly dependent on the genotype studied. *L. sativus* is perhaps a derivative from *L. cicera*, and *L. cicera* is in agreement with the reported phylogenic studies of *L. sativus* based on morphological and molecular markers (Chtourou-Ghorbel et al., 2001; Leht, 2009). However, green nodular calli induction was more difficult from explants of *L. cicera* than from those of *L. sativus* in the same medium. This difference might be related to the different ODAP levels between *L. cicera* and *L. sativus*. ODAP content is thought to be related to the stress resistance of *Lathyrus* species (Jiao et al., 2011a). Under drought stress, ABA could promote the accumulation of ODAP on leaves of *L. sativus* (Xiong et al., 2006), whilst the BA pretreatment might be regarded as a hormonal stress at high concentrations. Moreover, ABA causes decreases in callus induction and percentage of regeneration (Fazelienasab et al., 2004), and cytokinins are often considered as ABA antagonists in various processes in plants (Drüge &

Table 2. Comparison of the two *Lathyrus* species on induction rates of green nodular calli and the number of adventitious buds per explant.
Tabla 2. Comparación de las dos especies de *Lathyrus* en las tasas de inducción de callos nodulares verdes y el número de yemas adventicias por explante.

Variety	A	B	% of green nodular callus induction			No. buds per explant		
			Axillary bud	Stem	Leaf	Axillary bud	Stem	Leaf
		5	10.34 ± 3.18	3.55 ± 1.80	7.01 ± 1.12	6.2 ± 1.16	4.0 ± 0.71	1.8 ± 0.37
	0	10	29.86 ± 2.50	4.79 ± 0.26	5.17 ± 0.09	5.6 ± 1.03	3.5 ± 1.04	2.8 ± 0.66
		15	50.62 ± 6.08	4.81 ± 0.37	11.13 ± 0.36	5.8 ± 0.86	5.8 ± 0.86	3.25 ± 0.63
		5	25.26 ± 1.84	15.36 ± 1.64	23.04 ± 1.14	8.00 ± 4.04	6.25 ± 1.11	3.75 ± 0.85
	1	10	31.81 ± 1.94	19.48 ± 4.31	34.78 ± 4.50	8.28 ± 2.69	7.13 ± 0.64	4.13 ± 0.77
		15	64.24 ± 5.73	17.26 ± 0.99	33.88 ± 1.16	9.5 ± 1.04	7.25 ± 1.88	5.17 ± 0.91
		5	50.57 ±15.53	9.30 ± 0.99	22.72 ± 3.75	10.67 ±2.03	5.0 ± 1.00	3.67 ± 0.67
L. sativus	5	10	42.45 ± 6.09	8.16 ± 0.47	58.63 ± 5.46	7.57 ± 2.37	7.5 ± 1.18	6.0 ± 0.88
		15	66.55 ± 2.86	41.58 ± 5.87	65.3 ± 5.68	9.75 ± 2.18	6.18 ± 1.62	4.33 ± 0.71
		5	52.33 ± 2.31	17.86 ± 1.34	59.81 ± 1.06	6.5 ± 1.41	5.0 ± 1.53	2.57 ± 0.36
	10	10	56.53 ± 5.59	49.77 ± 9.55	65.53 ± 0.59	10.0 ± 2.35	6.73 ± 1.30	8.33 ± 2.24
		15	58.10 ± 2.21	43.52 ± 7.02	66.48 ± 2.10	6.0 ± 2.76	8.25 ± 3.99	5.29 ± 1.17
		5	75.03 ± 4.38	46.53 ± 8.59	38.89 ± 5.56	5.33 ± 0.73	3.29 ± 0.57	5.0 ± 1.16
	15	10	79.69 ± 4.36	39.52 ± 6.19	36.11 ± 7.35	7.08 ± 1.17	11.50 ± 2.67	8.75 ± 2.39
		15	82.30 ± 3.81	31.94 ±18.79	37.84 ± 7.72	4.89 ± 0.60	5.29 ± 1.21	7.0 ± 1.54
		5	33.53 ± 3.68	0	0	4.3 ± 0.88	/	/
	0	10	26.78 ± 2.01	0	0	3.67 ± 1.45	/	/
		15	34.74 ± 4.27	0	0	3.75 ± 0.63	/	/
		5	62.13 ±12.03	0	0	4.28 ± 0.94	/	/
	1	10	61.93 ±14.33	1.89	0	7.33 ± 1.49	/	/
		15	68.93 ±15.53	0	0	5.5 ± 1.02	/	/
		5	72.49 ±14.04	6.67 ± 1.67	13.59 ± 1.28	4.5 ± 0.57	3.75 ± 0.85	4.6 ± 1.21
L. cicera	5	10	71.36 ± 8.79	4.65 ± 2.36	18.23 ± 2.29	6.13 ± 0.85	4.25 ± 1.38	4.6 ± 0.93
		15	79.19 ±13.85	6.88 ± 0.81	16.70 ± 2.88	4.27 ± 0.65	6.75 ± 0.85	4.0 ± 0.84
		5	66.67 ±11.12	24.83 ± 1.46	36.05 ± 1.49	6.44 ± 1.52	6.33 ± 2.85	10.0 ± 1.30
	10	10	74.24 ±11.60	19.92 ± 1.85	31.15 ± 2.90	5.0 ± 1.07	4.67 ± 0.42	6.25 ± 0.75
		15	72.45 ± 7.11	22.20 ± 1.11	41.64 ± 1.11	5.75 ± 0.79	10.6 ± 1.89	5.89 ± 2.00
		5	77.62 ±11.30	49.99 ± 3.21	55.49 ± 4.35	5.5 ± 1.02	8.0 ± 1.78	4.38 ±0.49
	15	10	80.70 ± 5.87	49.14 ± 3.40	48.74 ± 1.73	8.18 ± 1.48	7.25 ± 1.11	4.82 ± 0.99
		15	86.59 ± 3.64	50.75 ± 2.66	62.13±10.71	6.67 ± 0.93	5.82 ± 1.02	3.25 ± 0.77

Note: A- BA concentrations of pretreated medium; B- BA concentrations of the induction medium for green nodular calli and adventitious buds.

Schonbeck, 1992). Therefore, *L. cicera* with low ODAP levels need high concentrations of BA to get the optimum. Interestingly, ODAP levels in leaf were the highest in the explants we used (Jiao et al., 2006), which also showed the most efficient in calli induction and shoot formation.

Effects of hormone type and levels on callus induction and shoot formation. From the three different hormone types (2,4-D, BA and NAA) tested for calli induction, BA appeared to induce green nodular calli regardless of the genotype. The optimum hormonal combination for regeneration was genotype-specific. All concentrations of BA tested in this experiment were effective for inducing meristematic nodular calli from leaf segments of *L. sativus*. However, only some BA concentrations were effective on *L. cicera*. Shoot development was increased with increases in BA concentrations up to a certain threshold, beyond which the frequency of shoot development was reduced (Table 2).

Several hormone combinations of auxin (it includes 2,4-D, NAA, IAA) and cytokinin (it includes BA, TDZ) have been used to induce the calli on *Lathyrus* species (Sinha et al., 1983; Roy et al., 1991, 1992; Zambre et al., 2002). Among them, 2,4-D and BA were the most effective and used frequently (Yang et al., 1992). When 2,4-D was used alone, however, nearly half of the calli varied in chromosome number or ploidy, and were difficult to differentiate further (Yang et al., 1992). Therefore, several studies added BA at a range of 4-22 μM to the MS medium for inducing adventitious shoot proliferation in *Lathyrus* and other plant species (Barik et al., 2005b; Xu et al., 2009). In this study, it is remarkable that BA induced green nodular callus, and subsequent shoot regeneration, regardless of the *Lathyrus* genotypes.

Rooting and hardening. About 2.0 cm long regenerated shoots were excised and cultured on half-strength MS medium with NAA (0.3, 0.5, 1.0 mg/L) for rooting. After 6 weeks, a good main root system and several lateral roots developed from the basal portion of the regenerated shoots on the two *Lathyrus* species (Fig 1H, 1P). However, the rooting efficiency was not exceeding 60% at any NAA concentration. This may be the result of the high cytokinin treatment at the induction period of adventitious roots (Xu et al., 2009). In fact, *Lathyrus* is very recalcitrant and difficult for rooting under *in vitro* conditions (Barpete et al., 2014b). These authors compared the effects of different auxins of NAA, IBA and IAA on rooting of grass pea. They found that while 2 mg/L IAA was the most suitable for rooting, the used IBA and NAA concentrations were not effective for development and growth of the lateral root system. These results are in agreement with ours; we also found that few lateral roots were formed when NAA was used.

Plantlets with well-developed roots were then washed thoroughly with distilled water, transplanted into pots containing water-saturated vermiculite and placed in the greenhouse with a 16-h photoperiod at 25 ± 1 °C. After 1 month, most of the regenerated plantlets (above 85%) survived and exhibited normal development. Several substrates had been used successfully in the transplanting of *Lathyrus* species (Barpete et al., 2014b). These researchers considered that substrates with light texture, adequate nutritive quality, good moisture-retention capacity and good aeration are necessary for an appropriate growth of the regenerated plantlets (Barpete et al., 2014b).

At present, several successful regeneration procedures are available in the genus *Lathyrus*. However, few of them have been used for the production of *Agrobacterium*-mediated transgenic plants. In the present study, two *Lathyrus* varieties with high and low contents of the neurotoxin ODAP were tested for their regeneration capacity. Using sterile seedlings pretreated with BA, an *in vitro* shoot regeneration protocol was established from different explants. This protocol might be helpful for plant improvement through either *Agrobacterium*-mediated genetic transformation or *in vitro* mutation breeding for reducing or eliminating the neurotoxic amino acid β-ODAP of *Lathyrus* species.

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REFERENCES

Barik, D.P., S.K. Naik, U. Mohapatrau & P.K. Chand (2004). High-frequency plant regeneration by *in vitro* shoot proliferation in cotyledonary node explants of grasspea (*Lathyrus sativus* L.). *In Vitro Cellular & Developmental Biology-Plant* 40: 467-470.

Barik, D.P., U. Mohapatra & P.K. Chand (2005a). Transgenic grasspea (*Lathyrus sativus* L.): Factors influencing *Agrobacterium*-mediated transformation and regeneration. *Plant Cell Reports* 24: 523-531.

Barik, D.P., U. Mohapatra & P.K. Chand (2005b). High frequency *in vitro* regeneration of *Lathyrus sativus* L. *Biologia Plantarum* 49: 637-639.

Barna, K.S. & S.L. Mehta (1995). Genetic transformation and somatic embryogenesis in *Lathyrus sativus*. *Journal of Plant Biochemistry and Biotechnology* 4: 67-71.

Barpete, S. (2015). Genetic associations, variability and diversity in biochemical and morphological seed characters in Indian grass pea (*Lathyrus sativus* L.) accessions. *Fresenius Environmental Bulletin* 24: 1-6.

Barpete, S., N.C. Sharma & S. Kumar (2014a). Assessment of somaclonal variation and stability *in vitro* regenerated grass pea plants using SDS-PAGE. *Legume Research* 37: 345-352.

Barpete, S., K.M. Khawar & S. Özcan (2014b). Differential competence for in vitro adventitious rooting of grass pea (*Lathyrus sativus* L.). *Plant Cell, Tissue and Organ Culture (PTOC): Journal of Plant Biotechnology* 119: 39-50.

Barpete, S., M. Aasim, K.M. Khawar, S.F. Özcan & S. Özcan (2014c). Preconditioning Effect of Cytokinins on *In Vitro* Multiplication of Embryonic Node of Grass Pea (*Lathyrus sativus* L.). *Turkish Journal of Biology* 38: 485-492.

Campbell, C.G., R.B. Mehra, S.K. Agrawal, Y.Z. Chen, A.M. Abd EI Moneim, H.I.T. Khawaja, C.R. Yadov, J.U. Tay & W.A. Araya (1994). Current status and future strategy in breeding grass pea (*Lathyrus sativus*). *Euphytica* 73: 167-175.

Chtourou-Ghorbel, N., B. Lauga, D. Combes & M. Marrakchi (2001). Comparative genetic diversity studies in the genus *Lathyrus* using RFLP and RAPD markers. *Lathyrus Lathyrism Newsletter* 2: 62-68.

Drüge, U. & F. Schonbeck (1993). Effect of vesicular-arbuscular mycorrhizal infection on transpiration, photosynthesis and growth of flax (*Linum usitatissimum* L.) in relation to cytokinin levels. *Journal of Plant Physiology* 141: 40-48.

Durieu, P. & S.J. Ochatt (2000). Efficient intergeneric fusion of pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.) protoplasts. *Journal of Experimental Botany* 51: 1237-1242.

Fazelienasab, B., M. Omidi & M. Amiritokaldani (2004). Effects of abscisic acid on calli induction and regeneration of different wheat cultivars to mature embryo culture. News directions for a diverse planet: Proceedings of the 4th International Brisbane, Australia.

Gharyal, P.K. & S.C. Maheshwari (1980). Plantlet formation from callus cultures of a legume, *Lathyrus sativus* cv., LSD2. *Zeitschrift für Pflanzenphysiologie* 100: 358-362.

Jiao, C.J., Q.L. Xu, C.Y. Wang, F.M. Li, Z.X. Li & Y.F. Wang (2006). Accumulation pattern of toxin β-ODAP during lifespan and effect of nutrient elements on β-ODAP content in *Lathyrus sativus* seedlings. *The Journal of Agricultural Science* 144: 369-375.

Jiao, C.J., J.L. Jiang, L.M. Ke, W. Cheng, F.M. Li, Z.X. Li & C.Y. Wang (2011a). Factors affecting β-ODAP content in *Lathyrus sativus* and their possible physiological mechanisms. *Food and Chemical Toxicology* 49: 543-549.

Jiao, C.J., J.L. Jiang, C. Li, L.M. Ke, W. Cheng, F.M. Li, Z.X. Li & C.Y. Wang (2011b). β-ODAP accumulation could be related to low levels of superoxide anion and hydrogen peroside in *Lathyrus sativus* L. *Food and Chemical Toxicology* 49: 556-562.

Kendir, H., N. Sahin-Demirbag, K.M. Khawar & S. Ozcan (2009). *In vitro* plant regeneration from Turkish grasspea (*Lathyrus sativus*) using immature zygotic embryo explants. *Biotechnology & Biotechnological Equipment* 23: 1177-1180.

Kumar, S., G. Bejiga, S. Ahmed, H. Nakkoul & A. Sarker (2011). Genetic improvement of grass pea for low neurotoxin (β-ODAP) content. *Food and Chemical Toxicology* 49: 589-600.

Kumar, S., P. Gupta, S. Barpete, A. Sarker, A. Amri, P.N. Mathur & M. Baum (2013). Grass pea. In: Singh, M., Upadhyaya H.D. & I.S. Bisht (eds), pp. 269-293. Genetic and Genomic Resources for Grain Legume Improvement.

Kunjumon, A., V. Ramesh Kannan & Y.T. Jasrai (1996). Plant regeneration from leaf explants of three grain legumes on same medium. *Journal of Plant Biochemistry and Biotechnology* 5: 27-29.

Leht, M. (2009). Phylogeny of old World *Lathyrus* L. (Fabaceae) based on morphological data. *Feddes Repertorium* 120: 59-74.

Malik, K.A., S.T.A. Khan & P.K. Saxena (1992). Direct organogenesis and plant regeneration in preconditioned tissue cultures of *Lathyrus cicera* L., *L. ochrus* (L.) DC. and *L. sativus* L. *Annals of Botany* 70: 301-304.

McCutchan, J.S. (2003). Review: A brief history of grasspea and its use in crop improvement. *Lathyrus Lathyrism Newsletter* 3: 18-20.

McCutchan, J.S., P.J. Larkin, P.A. Stoutjesdijk, E.R. Morgan & P.W.J. Taylor (1999). Establishment of shoot and suspension cultures for protoplast isolation in *Lathyrus sativus* L. *Journal of Breeding and Genetics* 31: 43-50.

Mehta, S.L., K. Ali & K.S. Barna (1994). Somaclonal variation in a food legume – *Lathyrus sativus*. *Journal of Plant Biochemistry and Biotechnology* 3: 73-77.

Meijer, E.G.M. & W.J. Broughton (1981). Regeneration of whole plants from hypocotyl-root, and leaf derived tissue cultures of pasture legume *Stylosanthes guyanensis*. *Physiologia Plantarum* 52: 280-284.

Murashige, T. & F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 472-497.

Ochatt, S.J., E. Muneaux & C. Machado (2002). The hyperhydricity of *in vitro* regenerants of grass pea (*Lathyrus sativus* L.) is linked with an abnormal DNA content. *Journal of Plant Physiology* 159: 1021-1028.

Roy, P.K., K. Ali, A. Gupta, G.K. Barat & S.L. Mehta (1993). β-N-oxalyl-L-α, β diaminopropionic acid in somaclones derived from internodes explants of *Lathyrus sativus*. *Journal of Plant Biochemistry and Biotechnology* 2: 9-13.

Roy, P.K., G.K. Barat & S.L. Mehta (1992). *In vitro* plant regeneration from callus derived from root explants of *Lathyrus sativus*. *Plant Cell Tissue and Organ Culture* 29: 135-138.

Roy, P.K., B. Singh, S.L. Mehta, G.K. Barat, N. Gupta, P.B. Kirti & V.L. Chopra (1991). Plant regeneration from leaf disc of *Lathyrus sativus*. *Indian Journal of Experimental Botany* 29: 327-330.

Schestibratov, K.A., R.V. Mikhailov & S.V. Dolgov (2003). Plantlet regeneration from subculturable nodular callus of *Pinus radiate*. *Plant Cell Tissue and Organ Culture* 72: 139-146.

Sinha, R.R., K. Das & S.K. Sen (1983). Plant regeneration from stem-derived callus of the seed legume *Lathyrus sativus L. Plant Cell Tissue and Organ Culture* 2: 67-76.

Spencer, P.S., A. Ludolph, M.P. Dwivedi, D.N. Roy, J. Hugon & H.H. Schaumburg (1986). Lathyrism: Evidence for role of the neuroexcitatory amino acid BOAA. *The Lancet* 2: 1066-1067.

Te-chato, S. & M. Lim (2000). Improvement of mangosteen micropropagation through meristematic nodular callus formation from *in vitro*-derived leaf explants. *Scientia Horticulturae* 86: 291-298 .

Xiong, Y.C., G.M. Xing, F.M. Li, S.M. Wang, X.W. Fan, Z.X. Li & Y.F. Wang (2006). Abscisic acid promotes accumulation of toxin ODAP in relation to free spermine level in grass pea seedlings (*Lathyrus sativus* L.). *Plant Physiology and Biochemistry* 44: 161-169.

Xu, Q.L., Y.H. Xie, H. Ru, X. Hu, C.Y. Wang & X.Y. Wang (2009). Efficient plant regeneration *in vitro* from red leaf beet via organogenesis. *Russian Journal of Plant Physiology* 56: 546-550.

Yang, H.M., Q.X. Gao, X.L. Wang & G.L. Sun (1990). Chromosome variation in cultured tissues of *Lathyrus sativus* L. *Journal of Lanzhou University (Natural Science)* 26: 103-108 (in Chinese).

Zambre, M., P. Geerts, A. Maquet, M. Van Montagu, W. Dillen & G. Angenon (2001). Regeneration of fertile plants from callus in *Phaseolus polyanthus* Greenman (year bean). *Annals of Botany* 88: 371-/377.

Zambre, M., B. Chowdhury, Y. Kuo, M. Van Montagu, G. Angenon & F. Lambein (2002). Prolific regeneration of fertile plants from green nodular callus induced from meristematic tissues in *Lathyrus sativus* L. (grass pea). *Plant Science* 163: 1107-1112.