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INDIRECT ORGANOGENESIS OF FABA BEAN (*VICIA FABA* L. *MINOR*)

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Using four Polish *Vicia faba* L. *minor* cultivars (Bronto, Dino, Tibo, Nadwiślański) we obtained callus from epicotyl fragments collected from 7- and 14-day-old seedlings and from cotyledonary nodes of immature seeds. Callus induction efficiency varied from 81% to 97% depending on the origin of the explant. Shoots regenerated only from the cotyledonary nodes of all tested cultivars. On average, 50% of the explants grown on MS medium containing 1.0 mg dm^{-3} NAA, 0.5 mg dm^{-3} BAP, 0.25 mg dm^{-3} GA_3, 1.0 g dm^{-3} casein hydrolysate, 750 mg dm^{-3} inositol, 3% sucrose and 0.4% agar were able to regenerate shoots. The number of calluses regenerating shoots was highest from explants collected from fruiting nodes 6 to 9. Decapitation of donor plants increased the percentage of calluses regenerating shoots. On half-strength MS medium with 2 mg dm^{-3} NAA and on 1/2 MS alone, 11% of the shoots rooted; on 1/2 MS with 1 g dm^{-3} AC, 8.0% rooted. The regenerants were transferred to Perlite with Hoagland medium and acclimated. Ten percent of the regenerated plants survived the acclimation process, flowered and produced seeds.

Key words: Callus, immature seed, organogenesis, in vitro culture, *Vicia faba* L. *minor*.

INTRODUCTION

The faba bean (*Vicia faba* L.) is important for agriculture because of its high protein content and symbiosis with *Rhizobium* bacteria. Large-seeded faba bean types (*V. faba major*) with 1000-seed weight greater than 1 kg have been developed in South Mediterranean countries, China, Mexico and South America. Medium-seeded types (*V. faba equina*) have been developed throughout the Middle East and North Africa, with a major concentration in Egypt. Small-seeded types (*V. faba minor*) with 1000-seed weight less than 500 g are found in Ethiopia and North European agriculture. The primitive form *V. faba paucijuga*, with 1000-seed weight lower than 250 g, ranges from Afghanistan to India. Some incompatibilities have been detected in sexual crosses between these types but are always minor, sometimes unilateral, and do not discriminate between these groups (Duc, 1997).

Vicia faba is still considered recalcitrant to regeneration in vitro due to lethal tissue darkening from accumulation of phenol oxidation products which inhibit cell division, leading to tissue darkening, necrosis and finally death (Griga and Klenoticova, 1994; Hamdy and Hattori, 2006a; Bahgat et al., 2008). Difficulties in regeneration may be caused by the high sensitivity of this plant to in vitro conditions such as changes in the water regime, temperature or light in closed dishes, and explant isolation, which stimulates phenol oxidation. A basic limitation to gene-transfer techniques or protoplast fusion is the absence of a working regeneration technique for *V. faba* (Duc, 1997).

The problem of maintaining viable callus and plant regeneration from *V. faba* subspecies has been discussed in a limited number of studies which have concentrated on *Vicia faba* L. *major*, usually used in human nutrition, and *V. faba* L. *minor*, used mainly for animal feed. Callus cultures of faba bean were obtained from hypocotyl explants (Vanketeswaran, 1962; Mitchell and Gildow, 1975), embryo radicles (Grant and Fuller, 1968) and immature cotyledons (Cionini et al., 1978). Despite optimization of nutrient media and growth regulators, the callus induction rate was ~1% and these calluses quickly darkened and failed to regenerate plants. Using plumule tips and hypocotyl fragments without meristem tips, Pevalek et al. (1980) induced callus on 67% of the explants. Jelaska et al. (1981) did not succeed in regenerating plants using immature and mature embryos without roots and plumule tips on 16 dif-

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ferent media. Selva et al. (1989) regenerated shoots from one genotype from cotyledonary, apex and axillary buds; they also rooted but did not acclimate. Khalafalla and Hattori (1999) reported multiple shoot production from the cotyledonary node explants of eight faba bean genotypes of diverse geographic origin. Later, investigations by Hamdy and Hatorri (2006a,b, 2007) revealed that an earlier-developed regeneration system for faba bean did not work efficiently for other cultivars. The number of regenerated shoots from mature seed cotyledons or nodal segments was low and they grew very slowly. Bahgat et al. (2008) obtained higher regeneration efficiency. The ability to undergo embryogenesis depended on the explant (higher for shoot tips) and cultivar. There have been two attempts to regenerate plants using protoplast culture. In one experiment, 20% of the protoplasts were capable of cell division and 4% formed a colony or callus (Röper, 1981). In another, 40% of the protoplasts divided and regeneration frequency was 5–10%, but only one cultivar out of ten tested was able to regenerate plants and the entire procedure took 14 months (Tegeder et al., 1995).

Griga et al. (1987) was the first to obtain embryogenic calluses from two *V. faba minor* breeding lines, although somatic embryo development terminated at the late torpedo stage and the embryos tended to form only roots or callus. The embryogenic callus showed no symptoms of darkening or necrosis, probably because the tested lines had naturally low content of phenolics and tannins. Apical and axillary meristem culture of five faba bean genotypes differed in shoot formation depending on seed tannin content (Griga, 1988). The genotypes with lower tannin content, especially those with white flowers, had a higher frequency of shoot multiplication (to 47%) than genotypes with high tannin levels (7.3%). The shoots rooted but did not acclimate ex vitro. Complete plantlets were successfully grown in soil (Griga and Klenoticova, 1994) and regenerated from shoot tips via callus within one year of culture. Callus formed on immature zygotic embryos developed flower buds in vitro. In both cases, the flowering shoots ended their vegetative growth with flower and leaf senescence and did not produce seeds.

In this report we describe a method for regenerating fertile *Vicia faba* L. *minor* plants through indirect organogenesis. Epicotyl fragments of young seedlings and cotyledonary nodes were tested to establish the regeneration procedure.

MATERIAL AND METHODS

PLANT MATERIAL

We used four Polish faba bean cultivars differing in the endogenous tannin content of the seeds and in their type of growth: Bronto, Dino, Tibo, and Nadwiślański (supplied from the breeding stations in Kończew, Radzików, Kobylin and Sobiejuchy respectively). Bronto has 0.02% tannin, approximately one hundred times lower than in the other cultivars. Bronto, Dino and Nadwiślański are characterized as having nondetermined growth type, and Tibo as having determined growth type.

Experiment I used explants consisting of 3–5 mm epicotyl slices of cv. Nadwiślański obtained after mature seed germination and collected from 14-day-old seedlings grown in vitro.

Experiment II used 3–5 mm epicotyl slices of 7-day-old (Fig. 1a) and 14-day-old seedlings grown in vitro and cotyledonary nodes (embryos without cotyledons, shoot tips and root tips) from immature seeds collected from the developing pods of consecutive fruiting nodes of plants of all examined cultivars, which were grown in experimental field plots. Half of the cv. Nadwiślański plants were decapitated above the 6th fruiting node. The pods at green maturity stage were collected at the same time from all fruiting nodes 12–14 weeks after seed sowing, from the end of June to mid July.

CULTURE CONDITIONS

Experiment I: The mature seeds were surface-sterilized for 3 min in 96% ethanol and 30 min in 10% sodium hypochlorite and rinsed five times in sterile water. The seeds were germinated in a 1 l glass jar in deionized water solidified with 0.4% agar. After two weeks the epicotyls were cut into 3–5 mm segments. These epicotyl fragments were cultured on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.4% agar (plant cell culture tested, powder), pH 5.8. For callus induction and regeneration, 0, 0.25, 0.5, 1.0, 2.0 mg dm^{-3} gibberellic acid (GA_3), 100, 250, 500, 750, 1000 mg dm^{-3} casein hydrolysate, 100, 250, 500, 750, 1000 mg dm^{-3} inositol and combinations of 0, 0.5, 1.0, 2.0 mg dm^{-3} naphtaleneacetic acid (NAA) with 0, 0.25, 0.5 mg dm^{-3} benzylaminopurine (BAP), 0.5, 1.0, 2.0 mg dm^{-3} thidiazuron (TDZ) and 0.5, 1.0, 2.0 mg dm^{-3} picloram (PIC) were tested. All chemicals were purchased from Sigma-Aldrich.

Experiment II: Epicotyl fragments of 7- and 14-day-old seedlings were obtained in the same way as in experiment I. The cotyledonary nodes were isolated from immature seeds collected from pods disinfected for 3 min in 70% ethanol, 30 min in 10% sodium hypochlorite, 3 min in 0.03% mercuric chloride and rinsed five times in sterile water. Explants were cultured on MS medium containing 1.0 mg dm^{-3} NAA, 0.5 mg dm^{-3} BAP, 0.25 mg dm^{-3} GA_3, 1000 mg dm^{-3} casein hydrolysate, 750 mg dm^{-3} inositol, 3% sucrose and 0.4% agar, pH 5.8.

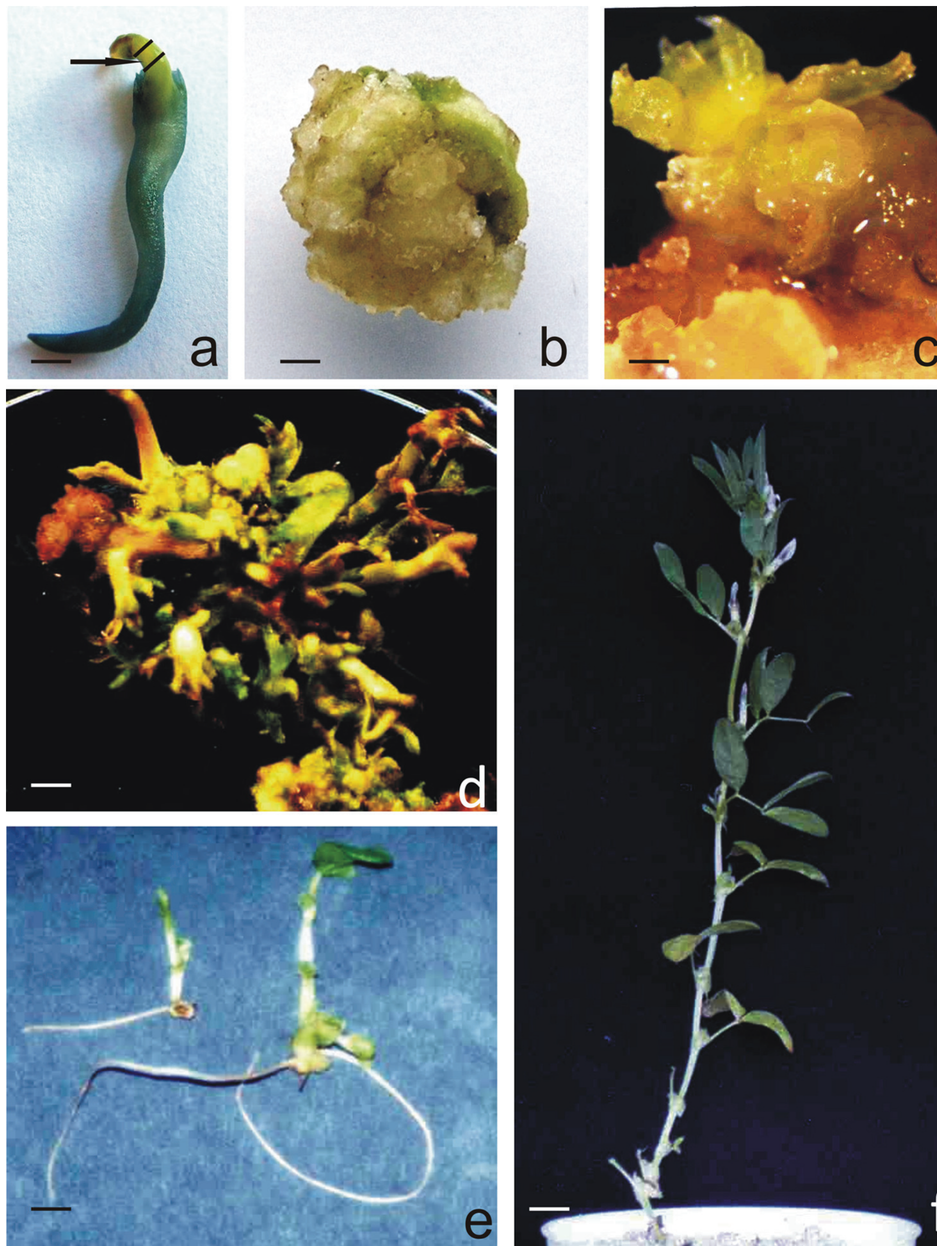

Fig. 1. Organogenesis in *Vicia faba* L. *minor* cv. Nadwiślański callus culture. (**a**) 7-day-old embryo with arrowed 3–5 mm slice used as explant. Bar = 1 cm, (**b**) Explant with callus on MS medium containing 1.0 mg dm^{-3} NAA, 0.5 mg dm^{-3} BAP, 0.25 mg dm^{-3} GA_3, 1.0 g dm^{-3} casein hydrolysate, 750 mg dm^{-3} inositol, 3% sucrose and 0.4% agar after 4 weeks of culture. Bar = 0.15 cm, (**c**) Callus starting shoot regeneration after 8 weeks of culture. Bar = 0.03 cm, (**d**) Shoot multiplication. Bar = 0.08 cm, (**e**) Rooted shoots on 1/2 MS medium with 2 mg dm^{-3} NAA. Bar = 0.5 cm, (**f**) Acclimated, flowering plant in pot. Bar = 7 cm.

All cultures were placed in a growth chamber under a 16 h photoperiod [240 μmol (quantum) $m^{-2}s^{-1}$] at 20°C. The cultures were transferred to fresh medium at 4-week intervals. Regenerated shoots were rooted in vitro on half-strength MS medium alone or with 2 mg dm^{-3} NAA or 1 g dm^{-3} activated charcoal (AC) and 0.4% agar, pH 5.8.

Rooted shoots were transferred to Perlite™ with Hoagland medium (Hoagland and Arnon, 1938) and acclimated to natural conditions. Then the plants were planted in soil and cultivated in greenhouse conditions until the seeds matured.

For all cultivars, each treatment consisted of 15 Petri dishes with 10–12 explants. During the process

TABLE 1. Induction and viability of *Vicia faba* L. *minor* cv. Nadwiślański callus on MS medium with different concentrations of BAP, TDZ, PIC and NAA after 8 weeks of culture

Growth regulator ($mg\ dm^{-3}$)		NAA ($mg\ dm^{-3}$)	Callus induction	Viable calluses (%)
BAP	0	0	*	0
		0.5	+	0
		1.0	+	0
		2.0	+	0
	0.25	0.5	++	0
		1.0	++	0
		2.0	++	0
	0.5	0.5	++	0
		1.0	++	23
		2.0	++	0
TDZ	0.5		++	0
	1.0	1.0	+	22
	2.0		+	0
PIC	0.5		+	0
	1.0	1.0	+	0
	2.0		+	0

* – no callus, + small callus mainly at cut parts of explant, ++ large callus covered whole explant.

of callus induction and regeneration we determined the fresh weight of calluses, number of regenerating calluses and number of shoots per callus.

STATISTICAL ANALYSIS

All data were statistically analyzed by ANOVA and Duncan's multiple range test or the t-Student test. P = 0.05 was taken to indicate significance.

RESULTS

Experiment I: Growth regulators, inositol and casein hydrolysate for callus induction were optimized using epicotyl fragments from 14-day-old seedlings. Callus induction (Fig. 1b) was observed on 85% of the explants. BAP with NAA and 0.5 $mg\ dm^{-3}$ TDZ with 1.0 $mg\ dm^{-3}$ NAA gave higher callus formation than the other combinations of TDZ and PIC with NAA (Tab. 1). Calluses cultured on medium with TDZ and PIC were compact and green with black fragments; calluses grown on medium with BAP and NAA were friable and yellowish. After 8 weeks of culture the calluses browned and did not regenerate. Only ~20% of them grown on medium with 0.5 $mg\ dm^{-3}$ BAP and 1.0 $mg\ dm^{-3}$ NAA or 0.5 $mg\ dm^{-3}$ TDZ and 1.0 $mg\ dm^{-3}$ NAA remained viable, and they did not regenerate either. Adding GA_3 to the medium with 0.5 $mg\ dm^{-3}$ BAP and 1.0 $mg\ dm^{-3}$ NAA increased callus viability to 66% (Tab. 2). Subsequent addition of inositol (Tab. 3) did not prolong callus viability, whereas casein hydrolysate (Tab. 4) not only gave higher viability but also helped to maintain it, for even sixteen weeks (data not shown).

TABLE 2. Viability of *Vicia faba* L. *minor* cv. Nadwiślański callus on MS medium with 0.5 $mg\ dm^{-3}$ BAP, 1.0 $mg\ dm^{-3}$ NAA and different concentrations of GA_3 after 8 weeks of culture

GA_3 ($mg\ dm^{-3}$)	Viable calluses (%)
0	23 b
0.25	66 a
0.5	66 a
1.0	30 b
2.0	29 b

Means with the same letter do not differ significantly at P = 0.05 by Duncan's test.

TABLE 3. Viability of *Vicia faba* L. *minor* cv. Nadwiślański callus on MS medium with 0.5 $mg\ dm^{-3}$ BAP, 1.0 $mg\ dm^{-3}$ NAA, 0.25 $mg\ dm^{-3}$ GA_3 and different concentrations of inositol after 8 weeks of culture

Inositol ($mg\ dm^{-3}$)	Viable calluses (%)
100	29 c
250	30 c
500	51 b
750	62 a
1000	39 c

Means with the same letter do not differ significantly at P = 0.05 by Duncan's test.

TABLE 4. Viability of *Vicia faba* L. *minor* cv. Nadwiślański callus on MS medium with 0.5 $mg\ dm^{-3}$ BAP, 1.0 $mg\ dm^{-3}$ NAA, 0.25 $mg\ dm^{-3}$ GA_3, 750 $mg\ dm^{-3}$ inositol and different concentrations of casein hydrolysate after 8 weeks of culture

Casein hydrolysate ($mg\ dm^{-3}$)	Viable calluses (%)
0	62 b
250	69 b
500	75 ab
750	82 a
1000	85 a

Means with the same letter do not significantly differ at P = 0.05 by Duncan's test.

TABLE 5. Effect of *Vicia faba* L. *minor* cultivar and type of explant on callus induction and fresh weight (g) after 8 weeks of culture (2E – epicotyl fragments from 2-week-old seedlings, 1E – epicotyl fragments from 1-week-old seedlings, C – cotyledonary node)

Cultivar	Callus induction (%)			Callus fresh weight (g)		
	2E	1E	C	2E	1E	C
Bronto	82 b	92 ab	95 ab	0.599 b	1.205ab	0.542 c
Dino	82 b	94 a	97 a	0.789 a	1.149 b	0.612 b
Tibo	81 b	89 b	92 b	0.815 a	1.230 a	0.502 c
Nadwiślański	85 a	95 a	98 a	0.810 a	1.300 a	0.689 a

Means with the same letter within column do not differ significantly at P = 0.05 by Duncan's test.

TABLE 6. Efficiency of *Vicia faba* L. *minor* callus regeneration obtained from cotyledonary nodes after 8 and 12 weeks of culture

Cultivar	Calluses regenerating shoots (%)		No. of shoots per callus
	8 weeks	12 weeks	
Bronto	15.5 b	56.0 a	2.5 b
Dino	16.7 b	52.4 ab	4.4 a
Tibo	25.4 a	50.0 b	4.2 a
Nadwiślański	23.8 a	57.7 a	4.5 a

Means with the same letter within column do not differ significantly at P = 0.05 by Duncan's test.

Experiment II: Fragments of 14-day-old epicotyls did not regenerate, so we took younger tissues to check their regeneration ability. Almost all explants cultured on MS medium with 1.0 mg dm^{-3} NAA, 0.5 mg dm^{-3} BAP, 0.25 mg dm^{-3} GA_3, 750 mg dm^{-3} inositol and 1000 mg dm^{-3} casein hydrolysate produced callus. Callus formation was highest, over 90%, on cotyledonary nodes from 7- and 14-day-old seedlings (Tab. 5). By week 8, despite the lower fresh weight of callus from cotyledonary nodes (Tab. 5), it was the only type of explant able to regenerate shoots (Tab. 6), which first appeared on the callus surface as small, compact green groups of cells and then developed into shoots (Fig. 1c). After 8 weeks of culture, 37.4–59.6% of the calluses were still deemed viable, without dark fragments. The cultivars differed in the percentage of callus regenerating shoots and in number of shoots. After 8 weeks of culture, 15.5–25.4% of the calluses were able to regenerate and 4 weeks later the regeneration rate increased to 50.0–57.7% (Fig. 1d). The average number of shoots was 2.5 for cv. Bronto to 4.5 for cv. Nadwiślański. The percentage of calluses regenerating shoots also depended on the fruiting node of plants and decapitation (Fig. 2). Donor plant decapitation considerably increased the percentage of calluses regenerating shoots, by 10–30% as compared with callus regeneration from nondecapitated plants. Explants taken from the upper part of the plant, above the 5th fruiting node, regenerated shoots ~1 cm longer than those from fruiting nodes 1–5. Multiple small shoots were regenerated almost equally for each fruiting node.

TABLE 7. Effect of NAA and AC on rooting of *Vicia faba* L. *minor* shoots regenerated in vitro.

Medium	No. of shoots for rooting	No. of rooted shoots*	Rooting [%]*
1/2 MS	200	22	11.0
1/2 MS + NAA	200	23	11.5
1/2 MS + AC	200	16	8.0

*mean value for all tested cultivars: Bronto, Dino, Tibo, Nadwiślański

We rooted the obtained shoots on 1/2 MS medium with 2 mg dm^{-3} NAA or with 1 g dm^{-3} or AC (Tab. 7). Root formation was observed only for shoots larger than 1 cm in the following percentages: 11.0% on 1/2 MS medium alone (Fig. 1e), 11.5% on 1/2 MS with NAA, and 8.0% on a medium with AC. The small multiple shoots failed to root.

Ten percent of the plants survived the acclimation process and transfer from in vitro to greenhouse conditions. These plants produced flowers (Fig. 1f) and formed pods with seeds. The derived seeds were smaller than those produced in vivo but were able to germinate.

DISCUSSION

An efficient, universal, genotype-independent regeneration system is necessary for genetic improvement of the faba bean, so that gene transfer can be added to the traditional breeding process. Here we described a system for regeneration via indirect organogenesis through callus, using cotyledonary nodes collected from the immature seeds of four Polish cultivars. Such an efficient method has not been fully elaborated before. The first reports focused mainly on selection of growth regulators and medium optimization to maintain viable callus in culture (Grant and Fuller, 1968; Mitchell and Gildow, 1975; Jelaska et al., 1981).

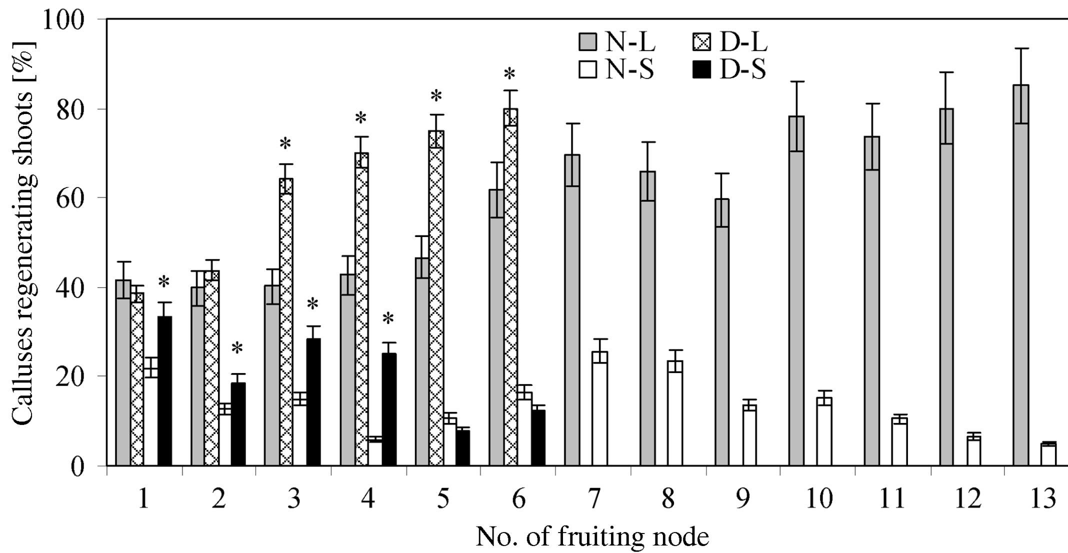

Fig. 2. Percentage of *Vicia faba* L. *minor* cv. Nadwiślański callus regenerating shoots, depending on the fruiting node and decapitation of donor plants. N – non-decapitated; D – decapitated donor plants; L – calluses with large shoots 1 cm long; S – multiple small shoots, difficult to count; * – significant difference in callus regeneration efficiency depending on decapitation of donor plants, at P = 0.05 by t-Student test.

Griga et al. (1987) made a breakthrough in faba bean culture in vitro when they described induction of somatic embryos in callus and suspension culture. However, the somatic embryos had a tendency to recallus or to form root-like structures on medium with 2,4-D, and embryogenesis was established only for faba beans with low tannin content. Shoot formation was obtained from cotyledonary, apex and axillary buds cultivated in the presence of BAP (Selva et al., 1989) or NAA and kinetin (Fakhrai et al., 1989). In this case the original position of the explants probably determined shoot induction due to residual apical dominance. Whole plant regeneration was first observed on medium supplemented with NAA and BAP, using as explants the immature zygotic embryos of two low-tannin lines (Griga and Klenoticova, 1994). Shoot multiplication was also achieved by culture of cotyledonary nodes of 7-, 14- and 21-day-old seedlings on medium supplemented with BA and TDZ (Khalafalla and Hattori, 1999) in seven cultivars from Africa, Asia and Europe. Shoot regeneration was also induced from cotyledon and nodal explants for six cultivars from other regions of the world (Hamdy and Hattori, 2006a,b). The adventitious shoot development percentage varied from 0 to 55.1% depending on the cultivar and on the type and concentration of growth regulators (Hamdy and Hattori, 2006a). Efficiency of regeneration was 1–2.5 shoots per callus (Hamdy and Hattori, 2006b). Bahgat et al. (2008) worked on a system for *Vicia faba* regeneration in two Egyptian cultivars using shoot tip and epicotyl explants. On medium with NAA, 2,4-D and high BAP content (10 mg dm^{-3}), 11–16% of the calluses derived from shoot tips produced embryos. On medium without 2,4-D and with 1 mg dm^{-3} BAP the shoot tip explants formed shoots directly and 2.25% of the calluses from epicotyl fragments produced somatic embryos. Within 8 months of formation of the first embryo, 400 shoots developed for cv. Giza 2 and 127 shoots developed for cv. 24 Hyto.

Almost all the mentioned publications described a more or less efficient method for shoot proliferation but not for rooting the shoots and cultivating them to mature plants. Only Khalafalla and Hattori (1999) described the regeneration responses of cotyledonary nodes as being equally efficient for all tested genotypes, and reported that 90% of the plantlets survived acclimation and were fertile. In our regeneration system, in addition to NAA and BAP we also supplied the medium with GA_3, inositol and casein hydrolysate, which increased callus viability and shoot regeneration to 57.7%. Importantly, regeneration was independent of genotype and tannin content, in contrast to other reports (Griga and Klenoticova, 1994; Khalafalla and Hattori, 1999; Hamdy and Hattori, 2006a,b; Bahgat et al., 2008). Our search for an explant responsive in regeneration ended in the finding that younger tissues and cotyledonary nodes isolated from immature seeds are more responsive than cotyledons or the apex and axillary buds used by other authors (Griga and Klenoticova, 1994; Khalafalla and Hattori, 1999; Hamdy and Hattori, 2006a,b; Bahgat et al., 2008).

In our experiments, shoots appeared on the surface of calluses within two months of culture initiation. In other work, shoots were regenerated within the same timeframe from nodal explants containing buds (Hamdy and Hattori, 2006a), or longer from other explants (Bahgat et al., 2008). We also found that decapitation of donor plants increased the number of calluses regenerating shoots, probably due to changes in endogenous levels of auxin and cytokinin in the shoots that were decapitated. According to Shimizu-Sato et al. (2009), decapitation of the shoot apex releases the axillary buds from dormancy and they begin to grow out. Auxin derived from an intact shoot apex suppresses axillary bud outgrowth, whereas cytokinin induced by decapitation of the shoot apex stimulates axillary bud outgrowth. We suggest that the inhibition of auxin polar transport after plant decapitation stimulates cytokinin synthesis and also transport to pods and seeds.

With proper selection of explants and optimization of the culture medium, in vitro micropropagation of faba bean can be carried out successfully. For the media described in this paper, faba bean regeneration was independent of the genotype. All tested cultivars regenerated shoots with more or less the same efficiency regardless of their tannin content and type of growth, whether determined or nondetermined. The plants acclimated to natural conditions and were fertile.

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