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In vitro propagation of *Vicia faba* L. by micro-cutting and multiple shoot induction

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Abstract. The influences of nitrogen sources, culture temperature and activated charcoal supplements were studied in relation to the rooting ability of *V. faba* cuttings. The interaction of these factors led to quantitative and qualitative modifications of the culture responses. Low temperatures (14–18 °C) were suitable for in vitro culture, limiting the formation of phenolics in plant material and making activated charcoal supplement unnecessary. Nitrogen supplements contributed in modifying the different plant responses, in accordance with temperature. Multiple shoot formation was obtained from the cotyledonary node and from the stem nodes cultivated in the presence of 6-benzylaminopurine (BAP). BAP at $4\,mg\,l^{-1}$ was the most effective concentration in promoting high rates of shoot development. The original position of stem nodes was found to determine the explant response to plant growth regulator treatments, possibly due to the effect of residual apical dominance.

Introduction

Grain legumes are economically important for their value as a protein source in human nutrition and animal feeding and for their role in biological nitrogen fixation [1]. Faba bean (*Vicia faba* L.) in particular is interesting as a protein and energy crop because of its adaptability to temperate climates and the high yield potentialities [2]. Unfortunately, susceptibility to environmental fluctuations results in yield instability, and unacceptance to growers. The possibility of overcoming this problem by breeding, with mass production of F1 hybrid seeds through the use of male-sterile lines [3, 4], remains hindered by the instability of this character in the rare mutants presently available [5].

The application of plant tissue culture, through protoplast culture and somatic hybridization, has been used to induce genetic variability and to create new nuclear-cytoplasmic combinations in several plant species [6]. However, the efficient use of these methods, particularly as a means of

manipulating male sterility, is subject to achievement of complete plant regeneration. The quality of the starting material has been emphasized as critical for protoplast culture and regeneration [7], particularly for recalcitrant species such as *V. faba*. In vitro propagated material that can (1) be available in large quantities, (2) persist in axenic condition on standardized media and (3) retain juvenile characters over long periods, is considered suitable for this purpose.

The present investigation examines these conditions in large-scale in vitro propagation of faba bean using micro-cutting and axillary bud proliferation techniques. The effects of nitrogen sources, temperature and charcoal supplement on the rooting and on the development of micro-cuttings, as well as the action of different growth regulators in inducing bud proliferation, are reported.

Materials and methods

Germination

Seeds of *Vicia faba* L. cv. Exelle and genotype Ad23 were obtained respectively from the Station d'Amélioration des Plantes, Ministère de l'Agriculture, Gembloux, Belgium and from the Station d'Amélioration des Plantes, INRA, Dijon, France. Exelle was used in micro-cutting experiments and Ad23 for axillary bud proliferation, although both genotypes proved to be equally responsive as micro-cuttings and propagated shoots (unpublished data).

The seeds were rinsed in 70% technical ethanol for 5 min and surface-sterilized for 30 min in commercial bleach supplemented with 1 drop of Tween 80. They were thoroughly rinsed three times with sterile distilled water and germinated on agar medium (0.8% w/v Bacto agar) in glass jars at 18 °C in the dark. After germination (5–7 days) seedlings were collected for experiments.

Micro-cuttings

Apical shoots (30–50 mm in height) were aseptically dissected and cultured in glass tubes (150 × 25 mm) sealed with transparent polycarbonate caps and containing 20 ml of culture medium. Basal medium contained half-strength MS [8] macro-elements devoid of nitrogen sources, micro-elements, vitamins [9], 2% w/v sucrose, 100 mg l^{-1} myo-inositol, 0.8% w/v agar (Bacto agar) and 10 g l^{-1} activated charcoal (AC). Nitrogen was added in

Table 1. Nitrogen supplements to micro-cutting basal medium.

Nitrogen sources ($mg\,l^{-1}$)	Medium				
	1.1	1.2	1.3	1.4	1.5
KNO_3	950	1960	950	–	–
NH_4NO_3	825	–	–	–	–
$(NH_4)_2SO_4$	–	–	–	1254	577

different combinations and concentrations as shown in Table 1. In a second experiment, AC was added to medium 1.3 in different concentrations (0, 5, 10, 15, 20 $g\,l^{-1}$). The pH of the media was adjusted to 6.5 with NaOH or HCl after the agar had been dissolved.

The experiments were conducted in a culture room with a light intensity of 1000 lux provided by 6 fluorescent tubes (Sylvania Gro-lux) under a 16 h photoperiod. Temperature was set at four different levels: 10, 14, 18 and 22 °C.

In each treatment, 42 apical shoots were cultured.

After 30 ± 1 days the numbers of rooted plants were scored. In addition, the number of adventitious roots, shoot length and number of distinct nodes were recorded for each rooted plant.

Multiple shoot formation from cotyledonary buds

Two types of explants, completely isolated buds and buds attached to half cotyledon were dissected from seedlings and cultured basal medium with the nitrogen supplement of MS [8]. 6-Benzylaminopurine (BAP) and 2,3,5-triiodobenzonic acid (TIBA) were added in various concentrations (0, 2, 4, 8, 16 $mg\,l^{-1}$) to the medium. Cultures were incubated in a culture room with a light intensity of 5000 lux supplied by 6 lamps Philips G/92/2:HPI/T 8M with a 16 h photoperiod at 18 °C. In each treatment, 37 explants were cultured. After 30 ± 1 days of culture the number of shoots per explant was counted.

Multiple shoot formation from apex and axillary buds

Shoot apices and axillary buds from all nodes were aseptically isolated from rooted cuttings obtained on medium 1.1 at 14 °C and separately cultured on the medium described for cotyledonary buds supplemented with 4 $mg\,l^{-1}$ of BAP and under the same experimental conditions. The number of proliferating shoots per explant was scored after each of the three culture periods (30 ± 1 days each).

Data analysis

Analysis of variance (ANOVA) and Duncan's Multiple Range test of SAS Institute Inc. statistical package were performed for balanced data sets. One-way analysis of variance and Tukey's Honestly Significant Difference (HSD) test of SPSS Inc. statistical package were used for unbalanced data sets.

Results and discussion

Micro-cuttings

Temperature and nitrogen source effects

Significant reductions in the number of rooted cutting occurred at 10 °C, displaying a clear-cut inhibitory effect on rooting ability (Table 2). Media 1.1 and 1.2 in particular amplified this effect while medium 1.4, containing a relatively high concentration of NH_4^+, appeared to partially overcome temperature inhibition. At 14 °C, when temperature was not limiting, medium 1.2 still reduced the rooting capacity. In the lower range of temperatures, a relatively high concentration of nitrate (1960 mg l^{-1}) as a sole nitrogen source appeared to enhance temperature inhibition.

The influence of temperature and nitrogen sources on rooting was also monitored by the number of adventitious roots, the shoot length and the number of distinct nodes. One-way analysis revealed significant effects ($P = 0.01, P = 0.001$) of temperature, media and their interaction (Table 3). Significant differences ($P = 0.05$) with the control (medium 1.1) were calculated for each temperature level with Tukey's test.

Table 2. Effect of culture temperature and nitrogen supplements on rooting of micro-cuttings. Number of shoots per treatment is 42.

Medium	Rooted shoots at temperatures of							
	10 °C		14 °C		18 °C		22 °C	
	Nr	%	Nr	%	Nr	%	Nr	%
1.1	24	59.5	40	95.2	39	92.8	42	100.0
1.2	21	50.0^{ns}	30	71.4**	40	95.2^{ns}	42	100.0^{ns}
1.3	33	78.6*	42	100.0^{ns}	42	100.0^{ns}	42	100.0^{ns}
1.4	35	85.7**	41	97.6^{ns}	42	100.0^{ns}	39	92.8^{ns}
1.5	28	66.6^{ns}	41	97.6^{ns}	42	100.0^{ns}	41	97.6^{ns}

*$P = 0.05$
**$P = 0.01$
^{ns}Not significant

Table 3. Effect of culture temperatures and nitrogen supplements on number of roots per explant, shoot length and number of nodes per shoot of rooted micro-cuttings.

Medium	Nr of roots/ explant (mean ± SD)	Range	Shoot length (mm) (mean ± SD)	Nr of nodes/ shoot (mean ± SD)
10 °C				
1.1	4.92 ± 2.26	1–8	68.78 ± 3.34	4.67 ± 0.96
1.2	3.48 ± 1.83^{ns}	1–7	74.19 ± 13.21^{ns}	4.62 ± 1.07^{ns}
1.3	4.67 ± 2.23^{ns}	1–10	62.06 ± 11.79^{ns}	4.24 ± 1.03^{ns}
1.4	4.43 ± 2.07^{ns}	1–10	62.71 ± 12.25^{ns}	4.26 ± 0.78^{ns}
1.5	3.46 ± 2.13^{ns}	1–9	68.20 ± 16.80^{ns}	4.61 ± 0.96^{ns}
14 °C				
1.1	9.73 ± 3.96	4–20	92.78 ± 19.92	4.53 ± 0.60
1.2	5.07 ± 2.43*	1–10	66.87 ± 16.20*	3.53 ± 0.82*
1.3	5.29 ± 1.70	2–10	65.43 ± 19.06*	5.55 ± 1.61*
1.4	5.44 ± 2.38*	1–10	68.88 ± 16.73*	5.07 ± 1.51
1.5	5.50 ± 3.04*	1–14	65.60 ± 19.52*	4.36 ± 1.68
18 °C				
1.1	5.15 ± 3.04	1–9	60.77 ± 20.70	2.26 ± 1.02
1.2	5.35 ± 2.16^{ns}	2–12	68.40 ± 21.79^{ns}	2.45 ± 0.93^{ns}
1.3	5.83 ± 2.54^{ns}	1–16	89.12 ± 29.23*	3.14 ± 1.32*
1.4	6.98 ± 2.56*	2–12	73.52 ± 23.33^{ns}	2.62 ± 1.10^{ns}
1.5	6.79 ± 2.29*	3–12	99.76 ± 14.37*	3.21 ± 0.56*
22 °C				
1.1	5.81 ± 2.23	2–11	83.26 ± 23.05	2.88 ± 0.80
1.2	5.24 ± 1.25^{ns}	2–8	89.48 ± 20.90^{ns}	3.00 ± 0.62^{ns}
1.3	4.64 ± 1.99^{ns}	2–10	86.36 ± 21.90^{ns}	2.86 ± 0.81^{ns}
1.4	6.00 ± 3.05^{ns}	2–18	83.85 ± 20.37^{ns}	3.08 ± 0.98^{ns}
1.5	5.17 ± 2.75^{ns}	2–12	89.17 ± 26.68^{ns}	3.37 ± 0.20^{ns}

*$P = 0.05$.
^{ns}Not significant.

Differences in nitrogen sources showed no significant influence on the qualitative traits of rooted cuttings compared with the control at 10 °C and 22 °C, whereas at 14 °C and 18 °C the effects of varying nitrogen sources were significant. At 14 °C, control medium 1.1 was the most favorable for multiple root initiation and stem elongation. Moreover, at 18 °C, ammonium-supplemented media were more effective in supporting multiple root initiation while a high level of nitrogen, either as NH_4^+ or as NO_3^- alone, was necessary to increase the stem elongation and the node development compared to medium 1.1. This indicated a possible competition in absorption of the two forms. The lower temperatures (10 and 14 °C), irrespective

of the medium, increased the number of nodes per plant without notably affecting stem elongation.

These results showed a predominant influence of temperature on plant architecture compared with nitrogen supplement, the latter showing an effect mainly within the range of intermediate temperatures (14–18 °C) which could be assumed to correspond to the thermic optimum for faba bean growth and development. The combination of a 14 °C temperature with medium 1.1 induced both differentiation of a high number of nodes and abundant development of adventitious roots (Fig. 1). The high number of nodes per plant increases the propagation rate through a second generation of cuttings or subsequent induction of shoot proliferation.

Nitrogen sources are known to play an important role in inducing in vitro growth and differentiation. Changes in the carbon/nitrogen ratio in culture media have been found to influence the production of phenolics in suspension cultures of rose and soybean [10]. Among the different concentrations

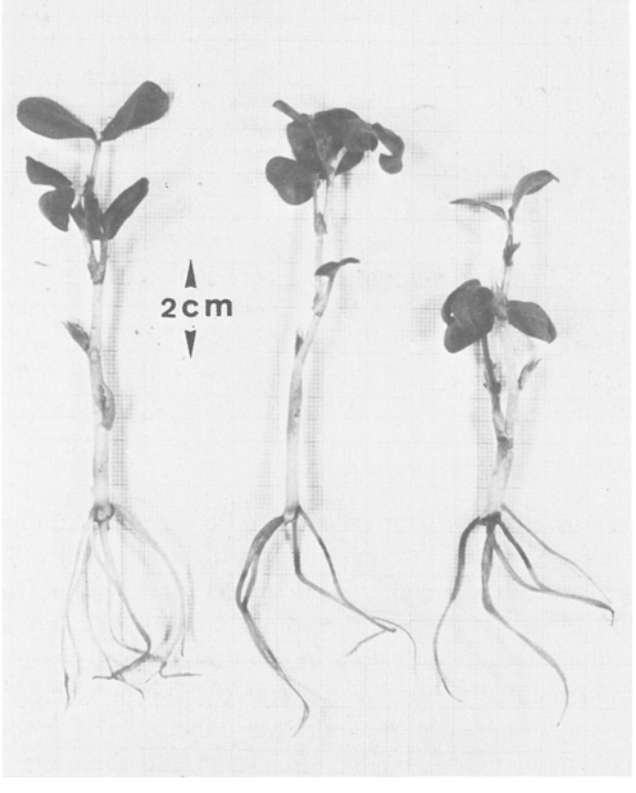

Fig. 1. Micro-cuttings rooted on medium 1.1 without AC at 18 °C.

and combinations tested, optimal callus growth was reported for *V. faba* in the presence of 20.6 mM NH_4NO_3 and 5 mM KNO_3 [11]. In contrast, improvements in inducing roots and in propagating meristems and calli were obtained by the reduction or exclusion of ammonium ions from the medium [12].

Our results indicated that the influence of nitrogen supplements is expressed mainly in correlation with temperature. No simple rule can be drawn from the data reported here, but the correct combination of these two factors may lead to the improvement of quantitative and qualitative characters expected for faba bean propagation in vitro. As a further approach, it will be interesting to study how the effects of the different culture regimes, and in particular of nitrogen sources [13], influence the isolation and culture of protoplasts.

Activated charcoal

Table 4 shows that in experiments conducted at low temperature (18 °C) the rooting frequency and number of adventitious roots were not increased by the presence of AC at any of the concentrations tested. There was no evidence of phenolic oxidation in any treatment, suggesting that the protective effect of AC is required only at the higher culture temperature (22–25 °C) reported by other authors [12, 14, 15].

The activated charcoal is known to absorb toxic substances, such as polyphenols produced by tissues during culture [16] and impurities present in the culture media [17]. On the other hand, AC absorbs auxins limiting its utilization [18]. In preliminary experiments with in vitro propagation via micro-cutting, high percentages of rooting were obtained with medium 1.1 at 22 °C. However, further utilization of this propagated material was limited by extensive blackening of tissues caused by the oxidation of phenolics. Similar findings have been reported to be characteristic of faba bean

Table 4. Effect of activated charcoal concentrations on micro-cutting rooting. Number of shoots per treatment is 32.

AC ($g l^{-1}$)	Rooted explants		Nr of roots/ explant (mean ± SD)	Range
	Nr	%		
0	32	100.0	6.59 ± 1.85	3–10
5	30	93.7^{ns}	5.56 ± 2.21^{ns}	1–9
10	32	100.0^{ns}	6.00 ± 2.79^{ns}	1–16
15	32	100.0^{ns}	5.84 ± 2.24^{ns}	2–13
20	31	96.9^{ns}	5.22 ± 0.08^{ns}	1–10

^{ns}Not significant.

cultured in vitro [12, 19, 20, 21] either at explant level or in indifferentiated cell cultures. Moreover, oxidation is enhanced in soft plant tissues by sterilization [12], limiting the source of starting material to seeds. Bieri et al. [22] overcome this problem by culturing shoot tips after disinfection at 4 °C for 4 days.

Our results emphasize that the temperature, among other factors, plays an important role in regulating growth responses and in improving plant quality.

Multiple shoot formation from cotyledonary buds

The concentration of BAP, the explant type (isolated bud and bud + half cotyledon), and their interactions have a highly significant influence ($P = 0.01$) on the number of shoots per explant (Table 5). In contrast, TIBA did not show any significant effect. Relatively low culture temperature (18 °C) was beneficial in improving shoot quality, as previously reported in this study for micro-cuttings.

These results indicate that the explant type has a strong influence in determining the number of developing shoots. The simultaneous presence of cotyledonary tissues and of BAP supplements appear to be necessary for the production of high number of shoots. The supplement of BAP to buds

Table 5. Influence of BAP concentrations and of the different explants on multiple shoot development.

BAP ($mg\,l^{-1}$)	Nr of shoots/explant (mean ± SD)	Range	Duncan's test				
			0	2	4	8	16
Bud + half cotyledon							
0	2.05 ± 0.70	1–3					
2	4.46 ± 1.56	1–9	**				
4	5.65 ± 1.06	3–8	**	**			
8	5.03 ± 1.74	1–8	**	*	*		
16	5.27 ± 1.60	2–9	**	**	ns	ns	
Bud							
0	2.40 ± 0.96	1–4					
2	2.73 ± 1.24	1–5	ns				
4	2.30 ± 1.29	1–5	ns	ns			
8	2.81 ± 1.29	1–6	ns	ns	*		
16	1.70 ± 0.91	1–4	**	**	*	**	

*$P = 0.05$ **$P = 0.01$ [ns]Not significant

dissected from the surrounding tissues failed to induce shoot proliferation and was inhibitory at the highest concentration ($16\,mg\,l^{-1}$). Howver, the cytokinin proved to be effective on the complex explant (bud + cotyledon). All of the concentrations tested induced a significant increment in the number of shoots. A supplement of $2\,mg\,l^{-1}$ of BAP was less effective than the higher concentrations, of which $4\,mg\,l^{-1}$ gave rise to the highest score (Fig. 2). Further increments in BAP concentrations did not increase the number of shoots.

Cytokinins are known to be effective in removing apical dominance [23]. A treatment with BAP promoted axillary bud growth in *Cicer arietinum* [24]. Multiple shoot proliferation has already been described in apical meristems [25, 26] and in axillary and cotyledonary buds cultures [27, 28] of grain legumes. BAP exclusively [22] or in combination with NAA [19] was reported to be effective in inducing bud proliferation on shoot tips of faba bean. Similar results were obtained by Schulze et al. [21] in cultures of shoot tips, nodes and hypocotyl axils of different *V. faba* genotypes on medium containing NAA and BAP. Hypocotyl axil gave rise to the highest number of shoots per explant (5–6 for the best genotypes), that where further successfully rooted on a medium supplemented with indoleacetic acid (IAA).

Our results are in agreement with reports by these authors in relation to the number of induced shoots on cotyledonary buds, only when part of the cotyledon is present on the explant. In contrast, the isolated bud appears to be less responsive to treatments.

A reduction of the BAP level of $2\,mg\,l^{-1}$ in further subcultures did not reduce the proliferation rate (data not shown), indicating the initial induction as the determinant event. Shoot elongation was observed also without auxin supplements, and regular removal of developed shoots promoted the growth of new ones. Excised shoots were rooted by subculturing on hormone-free medium.

Multiple shoot formation from apex and axillary buds

The one-way analysis of variance indicated a highly significant ($P = 0.01$) influence of the bud position on the number of proliferating shoots at three different times of continuous culture (Table 6). After the first culture period the presence of a residual apical dominance was still evident at the explant level, considerably limiting shoot proliferation in spite of the cytokinin supplement in the medium. This effect was most evident on shoot apex explants and became less important towards the lower node explants. During the second and third culture period, the residual apical dominance started to decrease, allowing the development of new shoots. Persistence of

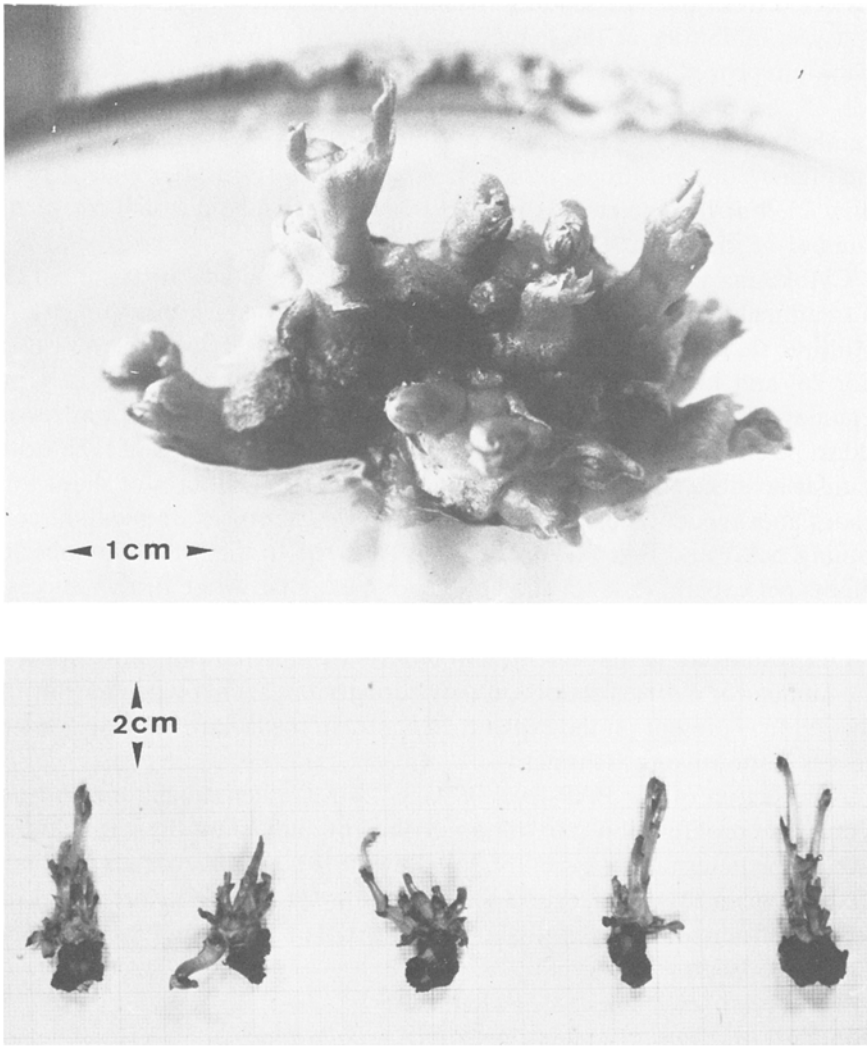

Fig. 2. Shoot proliferation from bud + cotyledon explants on basal medium with 4 mg l^{-1} BAP at 18 °C. *Top*: Buds developing on the explant after 2 weeks in culture. *Bottom*: Fully developed shoots obtained after 30 days in culture.

apical dominance in several explants (mainly shoot apices) during the first culture period led to the development of a single main shoot that inhibited the proliferation of other shoots.

This observation is confirmed by the percentage of explants still bearing only one shoot after the third culture period, i.e. 23% of the shoot apices in

Table 6. Influence of node position on multiple shoot induction.

Explant			No of explants	No of shoots/ explant (mean ± SD)	Range	Tukey's test				
						a	b	c	d	e
1st culture period										
a	Apex		94	1.04 ± 0.20	1–2					
b	Node	1	74	1.15 ± 0.36	1–2					
c	Node	2	77	1.71 ± 0.62	1–3	*	*			
d	Node	3	55	2.09 ± 0.48	1–3	*	*	*		
e	Node	4	21	2.14 ± 0.48	1–3	*	*	*		
2nd culture period										
a	Apex		94	1.89 ± 1.00	1–6					
b	Node	1	74	2.38 ± 0.77	1–4	*				
c	Node	2	77	2.92 ± 0.98	1–7	*	*			
d	Node	3	55	3.49 ± 0.98	1–6	*	*	*		
e	Node	4	21	3.90 ± 1.18	1–6	*	*	*		
3rd culture period										
a	Apex		94	2.67 ± 1.42	1–7					
b	Node	1	74	2.85 ± 1.04	1–6					
c	Node	2	77	3.40 ± 1.23	1–8	*				
d	Node	3	55	4.05 ± 1.43	2–9	*	*	*		
e	Node	4	21	3.81 ± 1.57	1–8	*	*			

*$P = 0.05$

culture. In contrast, for the other types of explant the percentage did not exceed 10%.

A way to make explants more sensitive to the cytokinin action is suggested by Saka et al. [26]. They found that shoot-bud formation on soybean stem node segments was markedly enhanced by preconditioning fresh tissues on a basal medium in the absence of growth regulators.

In conclusion, in vitro propagation of *Vicia faba* can be obtained by culturing the explants at low temperature (18 °C) limiting the formation of polyphenol oxidation products and modifying the plant response to nitrogen supplements. It has been found that 4 mg l^{-1} BAP was the effective concentration in promoting high rates of shoot development. A residual apical dominance effect was observed on the number of proliferating shoots in the presence of cytokinin.

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