Refinement of an *in vitro* Culture Technique for the Rescue of Globular Embryos Using Microcutting for *P. vulgaris* L. and *P. coccineus* L.

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Summary

Interspecific crosses between Phaseolus vulgaris *L. and* P. coccineus *L.(♀) almost always lead to embryo abortions at the globular stage. It is possible to rescue them using the pod culture technique, but this produces a very low regeneration rate (2.8%) due to poor root formation. In order to improve the regeneration of* P. coccineus *(♀) x* P. vulgaris *hybrid embryos, various tests were conducted with the aim of developing a micropropagation technique using microcuttings from cotyledonary nodes. The influence of the mineral composition of the culture medium and the addition of activated charcoal were evaluated as a means of promoting rooting. These tests served to evaluate the influence of the mineral composition of the culture medium and the addition of activated charcoal to the latter, in terms of encouraging rooting. The reaction of various genotypes to this technique was also evaluated. The use of a modified MS medium made it possible to produce plantlets with roots from 93% of cultivated explants of the NI16 variety of* P. coccineus*, while no regeneration took place when the plantlets were cultivated using a modified B5 Gamborg medium. It was possible to acclimate all plantlets produced on the modified MS medium. Micropropagation using microcuttings is more effective with* P. coccineus *(73.33% regeneration) than* P. vulgaris *(± 50%). The activated charcoal found in the modified MS medium made it possible to double the rate of regeneration from microcuttings in* P. vulgaris. *Pod culture combined with micropropagation using microcuttings from cotyledonary nodes achieved better results than regeneration methods using only pod culture. This new protocol opens up a new and promising method of rescuing embryos that would otherwise abort at the globular stage following interspecific hybridization using* P. coccineus *as the female parent.*

Résumé

Amélioration d'une technique de culture *in vitro* pour le sauvetage d'embryons globulaires par micro-bouturage chez *P. vulgaris* L. et *P. coccineus* L.

Les croisements interspécifiques entre Phaseolus vulgaris *L. et* P. coccineus *L.(♀) se soldent presque systématiquement par des avortements d'embryons au stade globulaire. Le sauvetage de ceux-ci est possible par culture de gousses mais avec un taux de régénération très faible (2,8%) suite à une mauvaise formation des racines. En vue d'améliorer la régénération d'embryons hybrides* P. coccineus *(♀) x* P. vulgaris*, différents essais visant à mettre au point une technique de micro-bouturage de nœuds cotylédonaires ont été réalisés. Ces essais ont porté sur l'évaluation de l'influence de la composition minérale du milieu de culture et de l'ajout de charbon actif dans celui-ci pour favoriser l'enracinement. La réaction de différents génotypes à cette technique a également été évaluée. L'emploi d'un milieu MS modifié a permis la production de plantules présentant des racines à partir de 93% des explants cultivés de la variété NI16 de* P. coccineus*; alors qu'aucune régénération de plantules n'a pu être obtenue en cas de culture des explants sur un milieu B5 de Gamborg modifié. Toutes les plantules produites sur le milieu MS modifié ont pu être acclimatées. Le micro-bouturage est plus efficace chez* P. coccineus *(73,33% de régénération) que chez* P. vulgaris *(± 50%). Le charbon actif dans le milieu MS modifié a permis de multiplier par deux le taux de régénération par micro-bouturage chez* P. vulgaris*. La culture de gousses combinée avec le micro-bouturage de nœuds cotylédonaires a permis d'obtenir des taux de régénération meilleurs que la culture de gousses seule. Ce nouveau protocole ouvre une nouvelle voie prometteuse de sauvetage des embryons qui avortent au stade globulaire lors des hybridations interspécifiques utilisant* P. coccineus *comme parent femelle.*

Introduction

Phaseolus vulgaris L. (common bean) is a leguminous crop that originated in Central and South America (10). It has the advantage of being a source of proteins (22% of seeds), cheap and contributing to food safety in high altitude regions in southern hemisphere countries (8). However, its average yield per hectare

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in 2008 was lower in Africa (0.61 t/ha) than in North America and the European Union (2 t/ha and 1.7 t/ha, respectively) (11). Biotic and abiotic constraints limit yields (18), particularly for small farmers in Africa and South America. Crossing *P. vulgaris* with two species from its secondary gene pool, *P. coccineus* L. and *P. polyanthus* Greenm., make it possible to improve its rusticity and resistance to diseases, but early abortions can be observed, especially if the common bean is used as a male parent (2, 6, 16). Embryo culture makes it possible to obtain hybrids from embryos at the late heart-shaped and cotyledonary stages (4, 9). In the majority of cases, abortion takes place at globular stage (23). Pod culture leads to the full development of globular embryos with *P. vulgaris* (NI 637 variety), but absence of root formation and stalled development of germinated embryos limits the proportion of growing plantlets to 2.8% of the total number of embryos produced by pod culture (14, 15). *In vitro* regeneration using explants other than the embryo has been reported in *P. vulgaris* and *P. coccineus* (3, 36, 37), using microcuttings from cotyledonary nodes from germinated seeds. However, this process is more effective with *P. coccineus* than *P. vulgaris* (36). The importance of the mineral solution (MS) (28) has been shown during regeneration from microcuttings of cotyledonary nodes with two *P. vulgaris* cultivars ("Lodino" and "Bico de Ouro") (3). On the other hand, the salts in the B5 medium developed by Gamborg *et al.* (12) are better than those found in the MS (28) used for regeneration from microcuttings of cotyledonary nodes with other *P. vulgaris* cultivars (Apetito G13637, Flor de Mayo Anita, ICA Palmar G4523 and Pinto Saltillo) (33). This difference could be explained by the existence of an interaction between the genotype and mineral composition of the microcutting medium for cotyledonary nodes. The addition of absorbent substances, such as PVP-360 (polyvinylpyrrolidone with a molecular weight of 360) or activated charcoal, encouraged rooting and *in vitro* seedling regeneration for *P. vulgaris* (5, 22). Based on these results, we aimed to develop an *in vitro* regeneration protocol from globular embryo by using microcuttings from cotyledonary nodes isolated from the stunted shoots found on *P. vulgaris* and *P. coccineus* germinated embryos. As part of this research, we tested the composition of the mineral solution used as a culture medium, the genotype and use of activated charcoal, for the regeneration of plants by micropropagation of microcuttings from cotyledonary nodes.

Materials and method

1. Plant material

The research was conducted at the Tropical Crop Husbandry and Horticulture Unit of the Gembloux Faculty of Agro-Bio Tech (GxABT) of the University of Liège. The *P. vulgaris* (NI 637 and X 484) and *P. coccineus* (NI 16) genotypes cultivated, which were used as plant material, were provided from the *Phaseolineae* collection maintained at GxABT – University of Liège.

2. Conditions for the culture of mother plants

The seeds are made to germinate in the dark in standard Petri dishes (Ø 9 cm), with 4-6 seeds in each dish. After 3-5 days of incubation, the germinated seeds are transferred to 1-litre pots, containing a mixture of 80% soil, 15% peat and 5% Rhine sand, to which 5-6 g of organic fertiliser are added (NPK)[1]. For 1-2 weeks, these pots are placed in conditioned cells until at least two trifoliate leaves appear and under the following climatic conditions: a day/night temperature of 24/20 °C, a relative humidity of ± 75%, a 12/12 hour day/night photoperiod with a light intensity of approx. 170 µmol. m^{-2}. s^{-1}. This measurement is taken 60 cm from the glass that separates the plants from 400-watt lamps. The young plants are then transferred to large polyethylene bags (3 L) containing the same substrate as the pots and placed in a larger growth chamber with identical climatic characteristics to those of the cells. The plants are watered twice per week with tap water, until they flower, which takes place after ± 30 days of culture for *P. vulgaris* and ± 45 days for *P. coccineus*. After flowering, nutrient solution is added each month (29).

Shoots are obtained from the parental genotypes after natural self-fertilisation for *P. vulgaris* or self-fertilisation for *P. coccineus*.

3. Pod disinfection

The freshly harvested pods are disinfected by immersion in an ethanol solution at 70° for 30 seconds, followed by calcium hypochlorite at 15 $g.L^{-1}$ for 1-3 minutes. They are then rinsed three times in sterile distilled water, in order to eliminate any trace of the disinfectant product. The part of the peduncle bleached by calcium hypochlorite is then removed prior to pod culture, in order to prevent the disinfectant spreading to the embryos.

4. Culture media

The germinated embryos, from which the cotyledonary nodes used as microcuttings are removed (Figure 1), were obtained from the young heart-shaped embryos isolated from pre-cultivated pods *in vitro*. For this purpose, the young pods (aged 5 days after pollination (DAP) for *P. coccineus* and 2 DAP for *P. vulgaris*), harvested from the plants and containing globular embryos, were cultivated in three media of decreasing molarity ($P_0$0 at 580 mosm, $P_0$1 at 450 mosm and $P_0$1 at 350 mosm) for one week, using the media described by Phillips *et al*. (31) and modified by Geerts (15). The young heart-shaped embryos isolated from pre-cultivated pods continue to develop until they

[1] Organic fertiliser NPK 6-3-12 + 2 MgO.

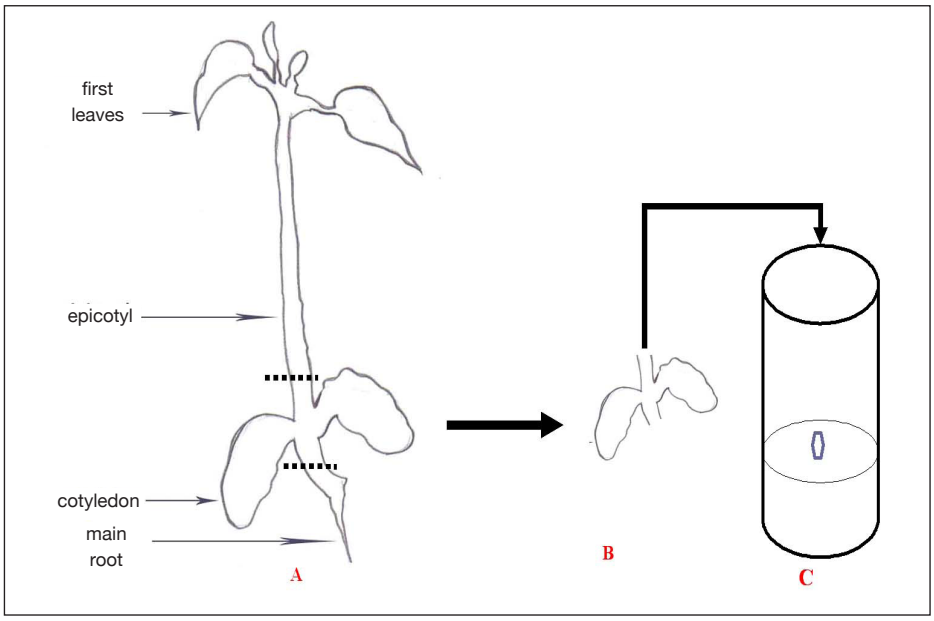

Figure 1: Schematic representation of microcutting.
Isolation of the cotyledonary node (A) followed by its transfer (B) to the culture tube containing a microcutting medium (C).

become germinated embryos (which should develop into plantlets) after being transferred successively to G1 media for maturation and germination (27) modified by the extraction of BAP, G6 for dehydration (19), G7g for root induction (15) and G7c for rooting or plantlet development (13, 15). These 4 culture media are prepared using the B5 mineral solution described by Gamborg *et al*. (12).
The appropriate culture medium for microcutting[2] was identified by evaluating the B5 mineral solution described by Gamborg *et al*. (12) in the G7c medium (13) and that of the MS (28) in a modified MS medium. The two media differ in terms of mineral salt composition, but are identical considering their vitamin, sucrose and hormone content and type of Agar used (13).
All the media were sterilised by autoclave at 121 °C for 20 minutes.

5. *In vitro* culture conditions

Conditions for the culture of pods and embryos isolated from the ovule have already been described in previous studies (13, 15, 27). For microcutting, culture tubes containing microcuttings and covered with transparent corks are placed in an incubator (Luminincube) under the following climatic conditions: temperature of 24 ± 2 °C, relative humidity of 100% inside the tubes, a 12-hour photoperiod and a light intensity of 60 $\mu mol.m^{-2}.s^{-1}$. These tubes are kept in the luminincube until the explants show vegetative growth.

6. Experimental protocol

6.1. Influence of the mineral solution

The culture of pods (13) harvested at 5 DAP was conducted with *P. coccineus* NI 16. This harvesting period corresponds to globular embryos (2). After one week in the G7c rooting medium, the explants are extracted from the tubes in order to remove the cotyledonary nodes (or microcuttings) (Figure 1). The microcuttings are cultivated separately in culture tubes using modified MS medium or G7c medium. After approx. 4 weeks of culture (the required time to regenerate a plantlet with one or more stems, at least one trifoliate leaf and many adventitious roots, each of the *in vitro* plants is transferred to a pot (Ø 6 cm) in order to be acclimated (27).
For each of the media tested, trial is made of 4 replicates and 55 microcuttings per replicate. The regeneration rates for *in vitro* plantlets and plants in acclimation are determined as a percentage of growing plantlets found in the total number of microcuttings transferred to tubes and the proportion of developed plants found in the total number of *in vitro* plants transferred to pots (Ø 6 cm), respectively.

6.2. Influence of the genotype

After the first microcutting test conducted using embryos of the NI 16 variety, a second test was conducted with two cultivated *P. vulgaris* genotypes (NI 637 and X 484). In this case, the pods are harvested 2 DAP, as this time corresponds to the globular stage of the embryos (16, 23). The *P. coccineus* genotype NI 16 acts as a control. After the cultivation of pods (15), all the microcuttings were transferred to the modified MS medium.
For each genotype, trials with three replicates were conducted with an average of 16 - 55 microcuttings per replicate according to the genotype. The numbers of plantlets generated *in vitro* are determined.

[2] The term “microcutting”, as used in this paragraph and the rest of the document, refers to the microcutting of cotyledonary nodes.

6.3. Influence of activated charcoal

On the basis of the immature heart-shaped stage reached by the embryos isolated from the pre-cultivated pods, we began this test with the culture of isolated young heart-shaped embryos (13). This makes it possible to overcome contamination problems affecting cultivated pods during this test period. For this reason, these embryos were isolated from the freshly harvested pods between 7-8 DAP for X 484 of *P. vulgaris* and between 9-10 DAP for NI 16 of *P. coccineus* (control). The isolated microcuttings (Figure 1) were transferred to tubes containing the modified MS medium, in the presence or absence of activated charcoal (0,5%). For each treatment, tests with five replicates were conducted on 50 microcuttings per replicate. The effectiveness of the activated charcoal was assessed according to the rooting rate and number of regenerated plantlets. The method used to determine the number of regenerated plantlets has already been described above. The rooting rate is determined according to the percentage of explants that developed adventitious roots with or without forming leafy stems.

7. Statistical analysis of results

The average variables observed are determined from the 3 to 5 replicates per trial, using Minitab software (version 14). The statistical analyses of the results are completed using the SAS programme (ANOVA) and the averages are classified using the Newman and Keuls test with a threshold of 5%.

Results

1. Influence of the mineral solution

The test used to determine the influence of the mineral composition of the modified MS medium and G7c medium on the success of the microcutting was conducted using cotyledonary nodes from embryos of the NI 16 variety of *P. coccineus*. Two to three weeks after the microcuttings were first cultivated, 100% bud break was observed in the latter with both media. For 204 out of 220 microcuttings (± 93%), the broken buds in the modified MS medium continued to grow and develop leafy and rooted stems after 4 weeks (Figure 2A). However, no plantlets were regenerated on G7c. This second medium caused a brown callus to appear on the part of the cutting that was in contact with the medium (Figure 2B). All the regenerated plantlets in the modified MS medium continued to develop into adult plants (producing flowers and shoots) after 45 days of acclimation.

2. Influence of the genotype

The regeneration of some cultivated microcuttings was observed in all the genotypes tested after 4 weeks (Table 1). However, the results obtained differ according to the genotype cultivated. The percentage of plantlets regenerated with the NI 16 variety of *P. coccineus* is greater (± 93%) than that obtained with genotypes X 484 and NI 637 of *P. vulgaris* (± 77%). A brown callus can be observed on the base of the *P. vulgaris* microcuttings that failed to develop into plantlets.

3. Influence of activated charcoal

The rooting problems encountered with *P. vulgaris* prompted us to test activated charcoal in the modified MS microcutting medium. The *in vitro* rooting and

Figure 2: Development of *P. coccineus* (NI 16) microcuttings after 4 weeks in the MS (A) and G7c (B) media.

A: Plantlet developed in the modified MS (28) medium; B: Stunted shoots with the formation of a brown callus on the root pole (blue stains) obtained using microcuttings cultivated in G7c (13) medium.

Table 1
Average number of regenerated plantlets as a percentage (%) according to genotype

Genotype	Total number of explants cultivated		Average number of developed plantlets (% of total)	
	Embryos	Microcuttings	Microcuttings	Embryos
NI 16	225	165	93.03 (± 3.60) a	73.33 (± 10.7) a
NI 637	108	48	76.59 (± 6.11) b	44.45 (± 4.81) b
X 484	108	60	77.32 (± 10.42) b	55.55 (± 4.81) b

The average numbers followed by identical letters are broadly equivalent to the probability threshold P≤ 0.05. The values between brackets refer to the standard deviations from the averages calculated after 3 replicate tests with a total of 16-55 microcuttings per replicate test depending on the genotype.

Table 2
Average rooting and plantlet development rates as a percentage (%) based on the presence of activated charcoal with *P. vulgaris* (X 484) and the control *P. coccineus* NI 16

Species	Culture medium	Total number of microcuttings	Average rate as a percentage	
			Rooted explant (*)	Regenerated plantlet
P. vulgaris (X 484)	MS+AC	250	89.17 a (± 6.8)	89.17 a (± 6.8)
	MS-AC	250	52.92 b (± 8.0)	43.43 b (± 4.5)
P. coccineus (NI 16)	MS+AC	250	88.33 a (± 4.8)	82.50 a (± 9.1)
	MS-AC	250	93.75 a (± 5.3)	85.83 a (± 3.5)

The average numbers followed by identical letters are broadly equivalent to the probability threshold P≤ 0.05. The values between brackets refer to the standard deviations from the averages calculated after 5 replicate tests with 50 microcuttings per replicate. (*): Rooted explant with or without leafy stems; MS: modified medium described by Murashige and Skoog (28). AC: activated charcoal.

regeneration rates obtained with *P. vulgaris* X 484 clearly show that the presence of activated charcoal in the modified MS medium produces better results in terms of *in vitro* rooting and regeneration (Table 2).
In fact, the average rooting rate with activated charcoal (± 90%) is significantly greater than that obtained if activated charcoal is absent (± 53%). If activated charcoal is present, the plantlets obtained present a large quantity of whitish adventitious roots (Figure 3A). However, if this constituent is absent, approximately half the microcuttings stopped growing after sprouting axillary buds and are characterised by the presence of a brown callus in place of roots (Figure 3B). The results from the control, *P. coccineus* NI 16, are equivalent and satisfactory with or without activated charcoal (± 90%). However, the presence of activated charcoal caused leaf chlorosis on NI 16, indicating physiological disorders, which appear after the formation of leaves after 3-4 weeks of cultivation.

Discussion

Our results concerning the influence of the medium's mineral composition, the addition of activated charcoal and the genotype on the success rate for the regeneration of *P. vulgaris* and *P. coccineus* varieties confirm those obtained by various other authors. The MS medium is used for the regeneration of plants using explants produced from germinated *P. coccineus* seeds (37). The importance of the MS mineral solution has also been shown for regeneration from microcuttings of cotyledonary nodes isolated from the germinated seeds with two *P. vulgaris* cultivars ("Lodino"and "Bico de Ouro") (3). These authors actually achieved better *in vitro* regeneration with the complete MS, compared to the MS medium, in which the nitrogen salts are reduced by one quarter. The latter medium ultimately causes necroses to appear and the formation of a callus at the base of the microcuttings, which come into contact with the medium, as we observed with the B5 salts in the medium described by Gamborg *et al*. (12). Developing shoots were seen on the axillary buds on cotyledonary nodes, which were kept intact on

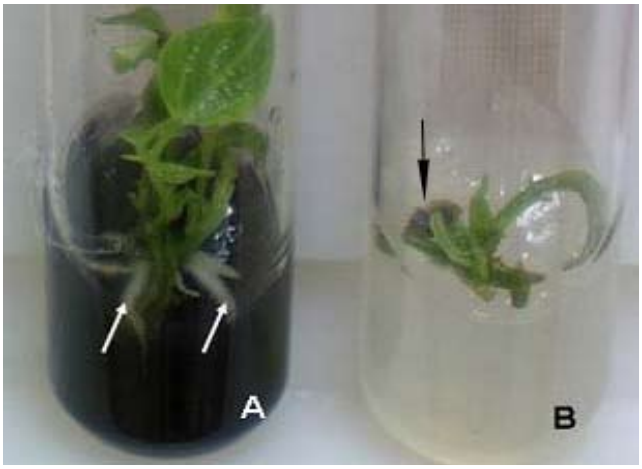

Figure 3: Plantlet (A) and explant (B) developed after 4 weeks of the microcutting of cotyledonary nodes using *P. vulgaris* (X 484), respectively, with or without activated charcoal.

A: Development of adventitious roots (indicated by white arrows) in a medium containing activated charcoal; B: Absence of regenerated plantelts without activated charcoal. The asterisk (*) indicates browning on the section of the explant, which comes into contact with the culture medium.

germinating embryos in an MS medium, using various leguminous food crops. This was the case with *Lens culinaris* Medik, taken from a culture of immature heart-shaped embryos (32), *Cajanus* (25, 26) and *Trifolium* (34, 35) taken from a culture of embryos at the mature heart-shaped or cotyledonary stages. In our test, the culture was initiated using globular embryos present in the shoots, which are cultivated *in vitro* after being removed from the plant. The sound growth of the shoots associated with the mineral salts contained in the MS medium could be linked to its nitrogen compound content (NH_4NO_3), which is higher than that of the B5 described by Gamborg *et al.* (12). The nitrogen is used for the synthesis of vital molecules for plant growth and development, such as amino acids, proteins, nucleic acids, nucleotides, chlorophylls and coenzymes (17). On the other hand, the opposite results were reported for the Flor de Junio Marcela, Apetito G13637, ICA Palmar G4523 and Pinto Saltillo *P. vulgaris* cultivars, for which plantlets could be regenerated *in vitro* from microcuttings of cotyledonary nodes (from germinated seeds) in B5 medium, but not in the MS medium (33). It is therefore possible that an interaction exists between the genotype and mineral composition of the culture medium for successful micropropagation from microcuttings. It is, however, certain that the genotype influences the success rate of plant regeneration using microcuttings. In our studies, the *in vitro* regeneration rate was significantly greater for *P. coccineus* than for the two *P. vulgaris* genotypes cultivated. The superiority of *P. coccineus* over *P. vulgaris* had already been reported in various regeneration studies focusing on cotyledonary nodes isolated from germinated seeds (36). This genotypic variation has also been reported for the regeneration of plants using direct organogenesis from the apex of the embryonic axis of 10 *P. vulgaris* cultivars (22). According to these authors, the formation of calluses depends on the capacity of each cultivar to heal, during *in vitro* culture, the wounds caused when the explant was excised. These same authors (22) were able to reduce the formation of calluses *in vitro* by optimising the composition of the culture media, mainly in terms of the appropriate cytokinin and auxin content for each cultivar. This reduced appearance of calluses contributed to the development of adventitious shoots, rooting and regeneration for all the *P. vulgaris* cultivars tested (22). However, it must be noted that, in many studies, the formation of calluses is a precursor to rhizogenesis induction, as reported for olive trees (*Olea europaea* L.) (7), cotton plants (*Gossypium hirsutum* L.) (30) and *P. vulgaris* (5). According to these authors (5), rhizogenesis only takes place when an absorbent substance, in particular PVP-360, is added to the media before rooting, i.e. the callus induction and adventitious shoot development medium. The same result is obtained if another absorbent is added - activated charcoal (22). The beneficial effects of these two absorbent substances on rooting can be explained by their ability to absorb inhibitory substances produced and rejected by the explant into the medium. We evaluated the effect of activated charcoal in the microcutting medium in terms of promoting rooting. This substance enabled us to double the regeneration rate for rooted *in vitro* plantlets to ± 90%, compared to ± 43% without the addition of activated charcoal. In this latter case, explants that fail to regenerate (± 57%) are characterised by the presence of a brown callus and the medium in direct proximity to the explant takes on a brownish colour. The beneficial effects of activated charcoal during *in vitro* regeneration have been reported by many authors, particularly in the case of explants from germinated *P. vulgaris* (22) and *Vicia faba* L. seeds (1). The absence of rooting or shoot growth is due to the production by the explant of toxic and inhibitory chemical substances, such as 4-hydroxybenzyl alcohol, which has been identified during somatic and zygotic embryogenesis in carrots (20, 21). A second effect, demonstrated with the NI 16 variety of *P. coccineus*, is that activated charcoal can cause physiological disorders by absorbing useful elements for plantlet development, such as magnesium and iron needed for chlorophyll synthesis (24). Total shoot growth inhibition after the addition of 0.5% activated charcoal to the microcutting medium has been observed during the *in vitro* regeneration of *V. radiata* L. (39). In view of the problems likely to be caused by the addition of activated charcoal (17, 38), it is vital to determine in each case the optimum concentration that should be added.

Conclusion

The large-scale regeneration of *P. coccineus* and *P. vulgaris* plants has been achieved by using microcuttings from cotyledonary nodes removed from germinated embryos obtained from a preliminary phase of pod culture. The study of the effects of the mineral composition of the culture medium on microcuttings of the NI 16 variety of *P. coccineus* has made it possible to show that a medium containing MS salts is favourable for *in vitro* regeneration via microcutting. However, this process is more effective with *P. coccineus* than with *P. vulgaris*. The cessation of growth of sprouted buds and the absence of rooting in this second species are linked to the formation of a brown callus at the base of the explants. The extent of this problem is reduced by adding activated charcoal at the dose of 0.5% to the medium. This new protocol, which combines embryoculture and micropropagation using microcuttings, has permitted to increase considerably the plantlet regeneration rate obtained by cultivating pods of the NI 637 genotype of *P. vulgaris* (15). Pod culture followed by micropropagation using microcuttings from cotyledonary nodes could be applied for the rescue of embryos produced by interspecific crosses of *P. coccineus* (♀) and *P. vulgaris*, which abort at globular stage. As the beneficial effect, which we observed,

of adding activated charcoal for *in vitro* rooting and shoot growth has not been reported systematically in studies on the use of this substance for promoting successful development of microcuttings, it is necessary to determine the appropriate concentration of this product on a case-by-case basis.

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