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Rescue of early heart-shaped embryos and plant regeneration of *Phaseolus polyanthus* Greenm. and *Phaseolus vulgaris* L.

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In order to improve plant recovery from early heart-shaped *Phaseolus* embryos, several refinements were made to the embryo rescue technique developed previously in our laboratory. Using *P. polyanthus* Greenm. and *P. vulgaris* L. genotypes, a 20% increase in the germination rate was obtained by using Phillips salts and 25 µg·l^{-1} ABA. This germination medium improved the development of the embryos and increased the number of normal growing plantlets. After germination, a 2-week dehydration treatment significantly stimulated the development of heart-shaped embryos into plantlets. A high variability in terms of growth and development of the embryos was observed among the *P. polyanthus* genotypes. A 50% germination rate was obtained for the NI 429 genotype, compared to an average of 20% for the other genotypes.
Keywords. *Phaseolus*, interspecific hybrids, embryo culture, ABA, mineral content, culture media.

Embryoculture d'embryons cordiformes jeunes de *Phaseolus polyanthus* Greenm. et *P. vulgaris* L. Plusieurs améliorations ont été apportées à la technique de culture *in vitro* d'embryons cordiformes jeunes de *Phaseolus* développé précedemment dans notre laboratoire. En utilisant des génotypes de *P. polyanthus* Greenm. et de *P. vulgaris* L., une augmentation du taux de germination de 20 % a été obtenue sur un milieu contenant les sels de Phillips et 25 µg·l^{-1} d'acide abscissique. Les embryons cultivés sur ce milieu de germination se sont mieux développés et ont donné un nombre plus élevé de plantules d'aspect normal. Après la germination, la culture des embryons pendant deux semaines sur un milieu de déshydratation suivie de leur tranfert sur le milieu d'enracinement habituel a amélioré significativement le développement en plantules des embryons cordiformes. Une grande variabilité dans la germination des embryons a été observée au sein des génotypes de *P. polyanthus.* Le génotype NI 429 a montré un taux de germination de 50 % contre 20 % pour les autres génotypes.
Mots-clés. *Phaseolus*, hybrides interspécifiques, culture d'embryon, acide abscissique, teneur en éléments minéraux, milieu de culture.

1. INTRODUCTION

Growing *Phaseolus* interspecific hybrids often requires using embryoculture. This allows the rescue of aborting immature embryos and the recovery of mature plants suitable for breeding programmes. To date, however, success has been limited to embryos more than 8 days old that had reached at least the late heart-shaped stage (**Table 1**). The incompatibility barriers between *Phaseolus* species usually causes abortion of the globular or early heart-shaped stage embryos. This is the case for crosses between *P. polyanthus* and *P. vulgaris* (Lecomte, 1997).

In vitro rescue of very immature embryos requires a culture medium that can support their growth and development. The use of inadequate media results in embryo necrosis, callus formation or premature germination, which leads in turn to weak and unbalanced seedlings. Mergeai *et al*. (1997) optimised the mineral nutrient, sugar and amino acid content in culture media. They used a modified Gamborg *et al.* (1968) medium to develop 30% of the heart-shaped *P. vulgaris* embryos into plantlets.

In this paper we report on improvements based on the use of Phillips *et al.* (1982) mineral solutions and abscisic acid (ABA), on the effects of using a dehydration treatment described by Hu and Zanettini (1995) and on the importance of the suspensor during early embryogenesis.

2. MATERIALS AND METHODS

2.1. Plant material and growing conditions

From the *Phaseolineae* active collection held at the Faculté universitaire des Sciences agronomiques de

Table 1. Regeneration of interspecific hybrids within *Phaseolus* using embryoculture — *Régénération d'hybrides interspécifiques au sein de* Phaseolus *en utilisant l'embryoculture*.

Crosses (female x male)	**Age** (days after pollination)	**Stage**	**References**
P. vulgaris L. x *P. ritensis* Jones	16–23	LC	(**1**), (**14**)
P. vulgaris L. x *P. lunatus* L.	12–24	C–LC	(**2**), (**16**), (**18**)
P. vulgaris L. x *P. acutifolius* A. Gray	14–28	C–LC	(**3**), (**6**) ,(**7**), (**8**), (**9**), (**12**), (**15**), (**16**), (**19**), (**20**)
P. coccineus L. x *P. acutifolius* A. Gray	15–20	C–LC	(**4**), (**17**)
P. coccineus L. x *P. vulgaris* L.	15–20	C-LC	(**5**)
P. vulgaris L. x *P. filiformis* Bentham.	7–24	LC	(**11**), (**14**)
P. vulgaris L. x *P. angustissimus* A. Gray	16–23	LC	(**10**), (**14**)
P. polyanthus Greenman. x *P. vulgaris* L.	19–33	LC	(**13**)

(**1**) Braak & Kooistra (1975); (**2**) Mok *et al.* (1978); (**3**) Rabakoarihanta *et al.* (1980); (**4**) Alvarez *et al.* (1981); (**5**) Shii *et al.* (1982); (**6**) Prendota *et al.* (1982); (**7**) Pratt & Bressan (1983); (**8**) Thomas & Waines (1984); (**9**) Pratt *et al.* (1985); (**10**) Belivanis & Doré (1986); (**11**) Weilenmann de Tau *et al.* (1986); (**12**) Parker & Michaels (1986); (**13**) Camarena & Baudoin (1987); (**14**) Petzoldt & Dickson (1987); (**15**) Andrade & Jackson (1988); (**16**) Cabral & Crocomo (1989); (**17**) Ben Rejeb & Benbadis (1989); (**18**) Kuboyama *et al.* (1991); (**19**) Jung *et al.* (1992); (**20**) Mejia-Jimenez *et al.* (1994); LC = Late cotyledonar; C = Cotyledonar.

Gembloux (Belgium), we selected four *P. polyanthus* cultivars (NI 429, NI 519, NI 553 and NI 1340) on the basis of their flowering ability, and one *P. vulgaris* cultivar (Bico de Ouro or NI 637). The *P. polyanthus* material was planted in greenhouses and in controlled growth chambers, while the *P. vulgaris* cultivar (NI 637) was grown in growth chambers.

In the greenhouses, the day and night temperatures were kept at 21°C and 17°C, respectively, and the relative humidity was 80%. Daylight was supplemented with HPL 400 watt vapor mercury lamps in order to provide 12 h of light per day.

The growing conditions in the growth chambers were: 24°C/20°C day/night temperature, 60% relative humidity, 580 $\mu E \cdot m^{-2} \cdot sec^{-1}$ light intensity (measured at 60 cm from 40 watt Grolux lamps) and 11h30 daylength.

Seed scarification, humidification and pre-germination in Petri dishes were carried out before sowing in a standard substrate mix (1/3 sand, 1/3 peat and 1/3 compost). The plants were watered every day and a nutritive solution – modified CERA2A– (Otoul, Le Marchand, 1974) was applied every week from the 20th day after sowing.

2.2. Embryo rescue

Young pods were harvested 8 days after the pollination of NI 637 and 11 days after the pollination of the *P. polyanthus* genotypes.

Embryos at the early heart-shaped stage were cultivated according to the technique described by Mergeai *et al.* (1997). The pods were surface-sterilised in 70% ethanol for one minute, and then immersed in 5% calcium hypochlorite for five minutes and rinsed three times in sterile de-ionised water. The ovules were removed from the pods under sterile conditions and placed in drops of de-ionised water solution containing 120 $g \cdot l^{-1}$ sucrose and 1.75 $g \cdot l^{-1}$ agar. The embryos were isolated under a stereo microscope using sterile needles. A Pasteur pipette was used to blow the embryos out to the surface of the G1 medium with a drop of the de-ionised water solution. The G1 medium, which fosters the germination of immature embryos, contains

- the Gamborg *et al.* (1968) salts,
- 400 $mg \cdot l^{-1}$ NH4NO3,
- 30 $g \cdot l^{-1}$ sucrose,
- 1 $mg \cdot l^{-1}$ thiamin/HCl,
- 5 $mg \cdot l^{-1}$ nicotinic acid,
- 0.5 $mg \cdot l^{-1}$ pyridoxin/HCl,
- 100 $mg \cdot l^{-1}$ myoinositol,
- 1000 $mg \cdot l^{-1}$ casein hydrolisate,
- 1000 $mg \cdot l^{-1}$ L-glutamin,
- 0.028 $mg \cdot l^{-1}$ N6-benzylaminopurin (BAP)
- and 8 $g \cdot l^{-1}$ Difco agar.

The immature embryos were cultivated in darkness at 26°C for 10 days and then transferred to a growth chamber at 26°C with an 11h30 daylength ($60\ \mu E \cdot m^{-2} \cdot s^{-1}$).

The embryos were transferred to the R1 rooting medium after 20 days of *in vitro* germination. This medium is based on the G1 medium but contains

- 100 $mg \cdot l^{-1}$ L-glutamin,
- 100 $mg \cdot l^{-1}$ casein hydrolysate
- and 0.03 $mg \cdot l^{-1}$ gibberellic acid (GA3), and lacks NH4NO3.

Germination took place in 90 mm Petri dishes, and rooting occurred in 20 × 150 mm borosilicate glass

culture tubes containing 20 ml of the culture medium. The Petri dishes were sealed with polythene film and contained eight embryos isolated at the same early heart-shaped stage. To ensure the oxygenation of the plantlets, the culture tubes were covered with plastic caps but not sealed with a polythene film. The plantlets showing a good rooting system were hardened in Jiffy pots containing 1:1:1 vermiculite, sand and compost. They were placed in a closed glass box and watered with the Murashige and Skoog (1962) salt solution. After a 15-day growing period, the plantlets were transferred to a growth chamber with 24°C/20°C day/night temperatures and a 12h30 daylength (60 $\mu E \cdot m^2 \cdot s^{-1}$).

2.3. Experiment on mineral composition

In order to improve the germination medium currently used in our laboratory, we compared the development of the immature embryos of the five *Phaseolus* genotypes – four *P. polyanthus* cultivars (NI 429, NI 519, NI 553, NI 1340) and one *P. vulgaris* cultivar (NI 637) – on a P1 medium, which is similar to the G1 medium in terms of organic and hormonal compounds but contains the Phillips *et al.* (1982) minerals.

2.4. Experiment on growth regulators and dehydration

The hormonal composition of the basic P1 medium was assessed with the *P. vulgaris* variety NI 637. It was compared to three media totally free of BAP and containing the following growth regulators:

- 0.06 $mg \cdot l^{-1}$ GA_3 + 25 $\mu g \cdot l^{-1}$ ABA (as the P2 medium),
- 0.06 $mg \cdot l^{-1}$ GA_3 (as the P3 medium)
- and 25 $\mu g \cdot l^{-1}$ ABA (as the P4 medium).

After germination, the embryos were transferred either directly to the R1 medium described by Mergeai *et al.* (1997) or to a dehydration medium for two weeks before cultivation on the rooting medium. The dehydration medium, designated the D1 medium, is similar to the G1 medium but lacks the hormone and amino acid and contains 100 $g \cdot l^{-1}$ sucrose and 5 $g \cdot l^{-1}$ activated charcoal.

2.5. Parameters and statistical analysis

The presence of an intact suspensor was noted. Embryo length was measured just after transfer to the germination medium (Li) and again 10 days later (Lf) in order to calculate the growth rate (GR) of the embryos according to the formula: GR = (Lf-Li)/Li. The germination rate was determined after 10 days culture on the germination media. The development and germination of embryos were also morphologically assessed in terms of the presence of leaf primordia and/or elongated radicle. Plantlet survival rate was noted 50 days after embryo dissection.

All the Petri dishes containing germinating immature embryos were distributed randomly in the growth chamber. Replicates varied from 10 to 20, according to the flowering duration and pod setting of the donor genotypes. The behaviour of *P. vulgaris* NI 637 on the Mergeai *et al.* (1997) germination medium was used as the control.

The results are discussed on the basis of variance analysis using one or two classification criteria (medium and genotype).

3. RESULTS AND DISCUSSION

3.1. Influence of mineral composition on the germination of early heart-shaped embryos

The mineral salt composition of the germination medium is one of the most important factors influencing the success of *in vitro* rescue of immature embryos (Monnier, 1976). The usual mineral nutrients are those of Gamborg *et al.* (1968) and Murashige and Skoog (1962). Recent work on various food legume species (Phillips *et al.*, 1982; Muños Florez, 1996; Rajemison, 1996) has highlighted the potential interest of the Phillips *et al.* (1982) salt solution (P1 medium) for *in vitro* culture. Embryo germination was higher on the P1 medium than on the G1 medium (**Figure 1**). Depending on the genotype, the embryo growth rate was higher on either the first or the second medium. No interaction between genotypes and media was observed for the parameters considered in this trial ($F_{obs} = 0.912$).

Phaseolus embryos of different origins reacted similarly to the modifications of the *in vitro* culture conditions. The same observation was made by Rajemison (1996) and Mergeai *et al.* (1997) in experiments conducted to assess the reaction of *P. vulgaris*, *P. polyanthus* and *P. coccineus* L. genotypes to different germination media. As in the case of wheat (Matthys-Rochon *et al.* 1998), the ability to grow *in vitro* differed among the genotypes. NI 429 had a germination rate of 50%, whereas the mean germination rate of all the other cultivars was 20±6%.

Apart from improving germination, the Phillips *et al.* (1982) minerals also improved cotyledon development (**Figure 2**), as well as homogenous and normal embryo development. These improvements were confirmed by further experiments with *P. vulgaris* (unpublished data). The major differences in the media relate to Ca^{++} concentration (150 $mg \cdot l^{-1}$ $CaCl_2 \cdot 2H_2O$ in the Gamborg *et al.* medium, as opposed to 600 $mg \cdot l^{-1}$ in the Phillips *et al.* medium) and nitrogen salts (250 $mg \cdot l^{-1}$ KNO_3 and 400 $mg \cdot l^{-1}$ NH_4NO_3 in the

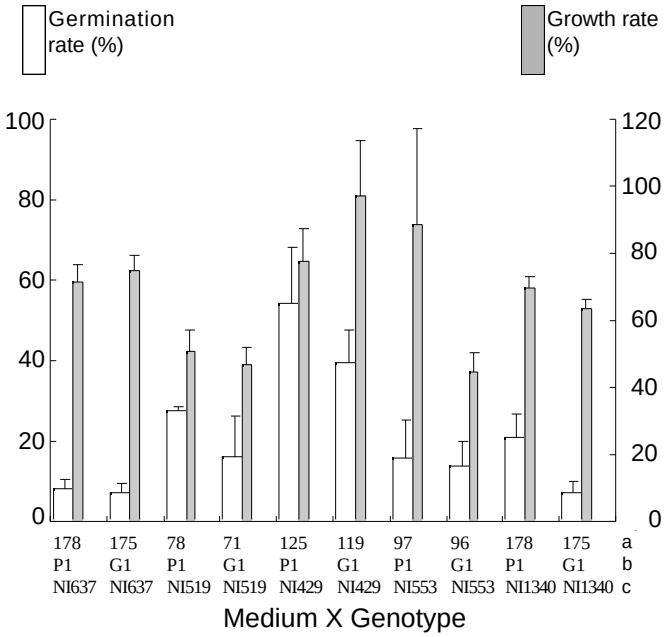

Figure 1. Average germination and growth rates of medium (P1 or G1) × genotype (NI 637, NI 519, NI 429, NI 553 or NI 1340) combinations. Bars represent the means (+ standard error, SE). a: number of embryos; b: medium; c: genotype — *Taux moyens de germination et de croissance pour chaque combinaison milieu (P1 ou G1) × génotype (NI 637, NI 519, NI 429, NI 553 ou NI 1340). Les barres représentent les moyennes (+ erreur standard, ES). a : nombre d'embryons; b : milieu; c : génotype.*

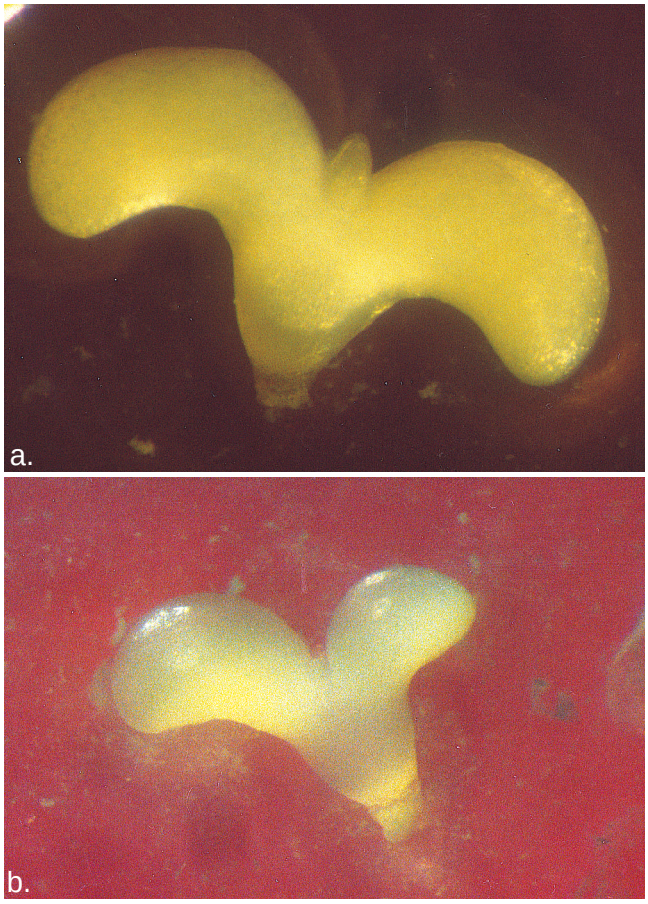

Figure 2. Development of *Phaseolus vulgaris* (NI 637) embryos grown on media containing the salts of Phillips *et al.* (1982) (photo a) or the salts of Gamborg *et al.* (1968) (photo b): embryos presented a more balanced development of their cotyledons on Phillips medium — *Embryons de* Phaseolus vulgaris *(NI 637) développés sur les milieux contenant les sels de Phillips* et al. *(1982) (photo a) ou les sels de Gamborg* et al. *(1968) (photo b): les embryons présentent un développement plus équilibré de leurs cotylédons sur le milieu de Phillips.*

modified Gamborg *et al.* medium, as opposed to 2100 mg·l^{-1} and 1000 mg·l^{-1} respectively in the Phillips *et al.* medium). These components are known to play an important role in embryo development. In *Capsella*, a high calcium content stimulated embryo survival by reducing the toxicity of other culture medium components (Monnier, 1976). As indicated by Denat *et al.* (1991), a reduction in calcium content inhibited *Helianthus* globular embryo development. Stewart and Hsu (1977) and Umbeck and Norstog (1979) demonstrated that the development of very immature embryos from cotton and barley, required a minimum level of reduced nitrogen due to their incapacity to reduce nitrate ions. Lecomte (1997) pointed out that the ammonium concentration of the Gamborg *et al.* (1968) medium is below the minimum required for the good development of young embryos. The higher concentration of ammonium in the Phillips *et al.* (1982) medium could therefore be a key factor in the improvement. However, the NO_3^-/NH_4^+ ratio also needs to be considered (George, 1993). In our Phillips *et al.* medium, this ratio was much lower than in the modified Gamborg *et al.* medium (2.66, as opposed to 4.71). According to several authors (Matsubara, 1964; Monnier, 1976; Liu *et al.* ,1993), a higher NO_3^-/NH_4^+ ratio is better for the *in vitro* development of early embryos. Our results do not concur with this view. A better knowledge of nitrogen nutrition during early embryogenesis in legumes could be of great interest.

3.2. Influence of growth regulators on embryo germination

Depending on the species and the age of the embryos, the choice of growth regulators and their concentration in the culture medium is a key factor in plant recovery from immature embryos (Raghavan, Torrey, 1964; Beasley, Ting, 1973; Monnier, 1976; Phillips *et al.*, 1982; Pellegrineschi *et al.*, 1997).

Studying the influence of different growth regulator concentrations in the germination medium described

by Mergeai *et al.* (1997), Lecomte (1997) observed that there was better embryo development when 0.06 mg·l^{-1} gibberellic acid (GA3) and 25 µg·l^{-1} abscisic acid (ABA) replaced 0.028 mg·l^{-1} N6-benzylaminopurine (BAP). The influence of growth regulators was assessed in a trial carried out with *P. vulgaris* (NI 637) heart-shaped embryos. The best results in terms of germination rate were observed in the P4 medium supplemented only with ABA, while the presence of GA_3 improved growth rates (**Figure 3**). The combination of GA_3 and ABAreduced the germination rate. Our observations also revealed precocious embryo germination and the development of weak and unbalanced plantlets on the GA_3-supplemented media (P2 and P3, data not shown). The positive effect of ABA on the germination rate has been reported for *Brassica napus* L. (Finkelstein, Crouch, 1986), *Zea mays* L. (Neill *et al.* 1987) and *Theobroma* sp. (Pence, 1992). Our results confirmed the ability of ABA to prevent the precocious germination of *Phaseolus* embryos reported by Prevost and Le Page-Degivry (1985). The same authors also showed that there were negative interactions between ABA and gibberellins or cytokinins. It is therefore not surprising that the replacement of BAP by ABA limited precocious germination considerably.

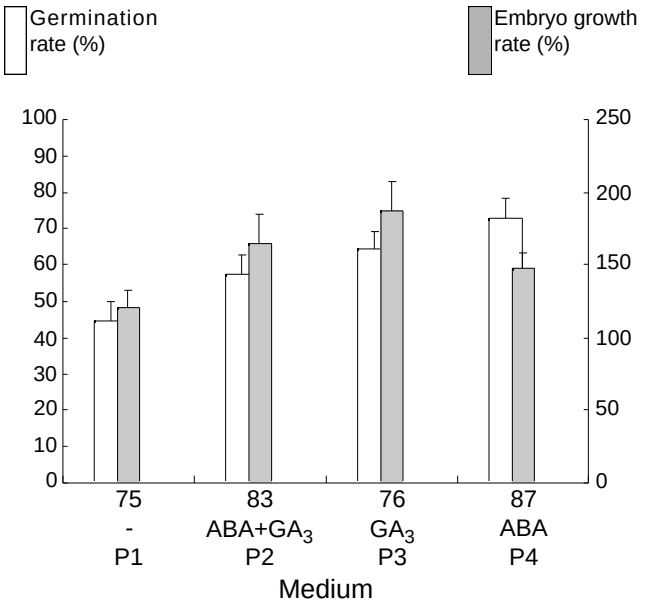

Figure 3. Germination and growth rates of *Phaseolus vulgaris* (NI 637) embryos for the four media tested. Bars represent the means (+SE). a: number of embryos — *Taux de germination et de croissance d'embryons de* Phaseolus vulgaris *(NI 637) pour les quatre milieux étudiés. Les barres représentent la moyenne (+ES). a : nombre d'embryons.*

3.3. Influence of germinated embryo dehydration before transfer to the rooting medium

In *Phaseolus*, as in soybean, rapid root growth initiated before shoot apex formation leads to hypocotyl swelling and unbalanced plantlets when germinating embryos are cultivated in a rooting medium. To improve embryo maturation and enhance plant development, Hu and Zanettini (1995) transferred germinating soybean embryos to a dehydration medium for two weeks before cultivating them on a rooting medium.

Our trial confirmed the positive effect of embryo dehydration on the subsequent survival of the plantlets (**Figure 4**). It showed that germination conditions clearly influence subsequent plantlet development. Most acclimatised plantlets were obtained from embryos germinating on the P2 and P4 media containing ABA. The influence of embryo dehydration was higher in presence of GA_3 than ABA. This could be explained by the inhibition of water uptake by ABA (Finkelstein, Crouch, 1986). High osmolality could therefore have an effect similar to that of ABA application on embryo development – it limits precocious germination. Similar observations have been made for *Brassica napus* L., *Zea mays* L. and the cacao-tree (Finkelstein, Crouch, 1986; Neill *et al.*, 1987; Cook *et al.*, 1988; Pence, 1992). The positive effects of ABA and embryo dehydration demonstrated the potential importance of water

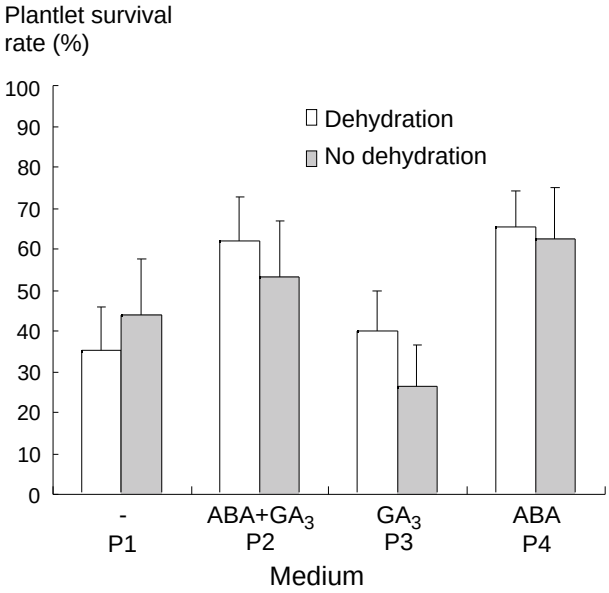

Figure 4. Influence of dehydration and germination media (P1–P4) on the survival rate of *Phaseolus vulgaris* (NI 637) plantlets. Bars represent the means (+SE). — *Influence de la déshydratation et des milieux de germination (P1–P4) sur le développement d'embryons germés de* Phaseolus. *Les barres représentent les moyennes (+ES)*.

relations between the ovule and the embryo at the beginning of zygotic embryogenesis. Interaction between osmotic pressure variation and ABA content has been confirmed by several research teams (Finkelstein, Somerville, 1989; Wilen *et al.*, 1990; Belefant, Fong, 1991; Salisbury, Ross, 1992; Liu & Kriz, 1996; Pacheco-Moisès *et al.*, 1997). An increase in these two factors stimulates protein storage (Sussex *et al.*, 1975; Barratt, 1986; Wilen *et al.*, 1990; Leal *et al.*, 1995) and triacylglycerols synthesis (Attree *et al.*, 1991; Pacheco-Moisès *et al.*, 1997), and regulates genes at the mRNA level (Hetherington, Quatrano, 1991; Hollung *et al.*, 1997).

3.4. Role of the suspensor

During our experiments, no embryo germinated when its suspensor was damaged during transfer to the culture medium. Yeung *et al.* (1980) and Brady and Comb (1989) have already demonstrated the active role of the suspensor in *Phaseolus coccineus* and *P. vulgaris*.

4. CONCLUSIONS

Success in embryo rescue using *P. vulgaris* was limited to embryos more than eight days old that had reached at least the heart-shaped stage. Although Smith (1973) and Lecomte (1997) succeeded in regenerating some adult plants from 6-day-old embryos, the plant regeneration rates they obtained remained relatively low (about 30%). In this study, we improved the mineral and hormonal composition of the culture medium to stimulate the growth and development of heart-shaped *Phaseolus* embryos. We also assessed the effects of dehydrating the germinating embryos before inoculating them in a rooting medium. Our results show that germination increased when using Phillips *et al.* (1982) minerals, and 25 $\mu g \cdot l^{-1}$ ABA + 0.06 $mg \cdot l^{-1}$ GA_3 as growth regulators (ABA alone giving the best results). The average germination rate reached 50%. Dehydration also stimulated subsequent development of the germinated embryos in rooting medium.

There was a very high variability among the *P. polyanthus* genotypes. Genotype NI 429 exceeded the germination rate of all the other cultivars by 30%. In addition, *P. polyanthus* genotypes showed a better germination rate than the *P. vulgaris* variety NI 637. More attention also needs to be paid to the suspensor during embryo transfer to the medium within *Phaseolus*. Indeed, no embryo germinated when its suspensor was damaged during transfer to the culture medium.

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