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EARLY ABORTION IN RECIPROCAL CROSSES BETWEEN *PHASEOLUS VULGARIS* AND *PHASEOLUS POLYANTHUS*, AND *IN VITRO* CULTURE OF IMMATURE EMBRYOS FROM THESE SPECIES

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ABSTRACT. — The causes of early embryo abortion in reciprocal crosses between *Phaseolus vulgaris* L. and *Phaseolus polyanthus* Greenm. were studied. Methacrylate resin sections, 2 µm thick, of 2 to 6 day-old hybrid seeds were used to examine the stages of embryo development and the state of seed tissues. These observations permitted to suggest the main causes of abortion and to identify the developmental stages at which hybrid embryos should be rescued. Early nutritional barriers in *P. polyanthus* (♀) × *P. vulgaris* crosses are related to a deficient endosperm development while in reciprocal crosses, endothelium proliferation and to some extent, hypertrophy of the vascular elements might be the main causes of early embryo abortion. The importance of the abnormalities observed during embryo development depended to a great extent on the compatibility between the genotypes crossed. Our results also suggest that the appropriate time for embryo rescue when *P. polyanthus* is a female partner is at the early globular stage. Several parameters of *in vitro* culture were analysed within the two parental species : genotype, culture medium, age of the collected pods. The results show that plantlets regeneration can be obtained satisfactorily in *P. vulgaris*, while the rate of success remains limited in *P. polyanthus*.

KEY WORDS. — *Phaseolus*, interspecific hybridisation, incompatibility barriers, embryo, *in vitro*, osmotic pressure.

RÉSUMÉ. — Les causes de l'avortement précoce des embryons résultant des hybridations interspécifiques réciproques entre *Phaseolus vulgaris* L. et *P. polyanthus* Greenm. sont étudiées. Des coupes semi-fines de 2 µm, réalisées dans des graines hybrides âgées de deux à six jours, enrobées dans une résine du type méthacrylate, ont permis d'analyser les stades de développement embryonnaire et l'état des tissus environnants. Les observations ont permis de proposer les principales causes de l'avortement et d'identifier les stades de développement auxquels les embryons hybrides pourraient être sauvés. Rapidement, des barrières nutritionnelles peuvent être observées dans les croisements *P. polyanthus* (♀) × *P. vulgaris* en raison d'un développement réduit de l'albumen, alors que dans les croisements réciproques, la prolifération de l'albumen et, dans quelques cas, l'hypertrophie d'éléments vasculaires constitueraient les causes principales de l'avortement précoce des embryons. L'importance des malformations constatées durant le développement embryonnaire dépend en grande partie de

la compatibilité entre les génotypes croisés. Les résultats suggèrent aussi le stade globulaire jeune comme le moment approprié pour le sauvetage des embryons hybrides lorsque *P. polyanthus* est le parent femelle. Plusieurs paramètres de la culture *in vitro* sont analysés pour les deux espèces parentales : le génotype, le milieu de culture et l'âge des gousses récoltées. Les résultats montrent que la régénération de plantules peut être obtenue de manière satisfaisante chez *P. vulgaris*, mais par contre que le taux de réussite reste limité chez *P. polyanthus.*

MOTS-CLÉS. — *Phaseolus*, hybridation interspécifique, barrière d'incompatibilité, embryon, *in vitro*, pression osmotique.

INTRODUCTION

Among the pulses, the common bean, *Phaseolus vulgaris* L., of neotropical origin, is one of the most important species, widely distributed in the world (FAO statistical data base 1998). In the developing countries, it is considered as a good source of protein, its seeds complementing the seeds of cereals in the nutritional value of daily diet. It is also considered as a cover and green manure crop. In addition, *Phaseolus* beans are adapted to several cropping systems, particularly in association with other food crops, like maize, sorghum or pearl millet (SINGH 1999). *Phaseolus* beans, in many cropping systems, particularly in the tropics, are characterised by low and inconstant seed yield. This is mainly due to the susceptibility of the crop to numerous pests and diseases. More than 200 pathogens, for example, have been reported attacking beans, some of them causing considerable economic losses (KORNEGAY & CARDONA 1991, GRAHAM & RANALLI 1997, SINGH 1999). Another constraint limiting the yield is the lack of improved varieties tolerant to abiotic stresses (poor soil, high temperature, drought, etc.) at the small farm level. Agronomists and breeders have not found sufficient genetic variability within the primary gene pool of *P. vulgaris*. However, the secondary gene pool (DEBOUCK & SMARTT 1995, DEBOUCK 1999) offers very good breeding potentialities. This is particularly the case with the two species *Phaseolus coccineus* L. and *Phaseolus polyanthus* Greenm. containing genes of resistance or tolerance to biotic and abiotic constraints (BAUDOIN *et al.* 1992, BAUDOIN 2001). Of special interest is the very high level of resistance to *Ascochyta* leaf spot, a fungus disease prevailing in highland areas, among *P. polyanthus* germplasm (SCHMIT & BAUDOIN 1992).

In crosses between *P. vulgaris* and *P. polyanthus,* the use of the common bean as mother plant increases the rate of successful crosses but, unfortunately, the presence of *P. vulgaris* cytoplasm provokes a quick reversal to the recurrent species, at the expense of the donor species. Reciprocal crosses are more difficult to perform, because of embryo abortion at a very early stage, 3 to 5 days after pollination (DAP). The very few hybrid plants obtained showed, however, better possibility of introgression (CAMARENA & BAUDOIN 1987, BAUDOIN *et al.* 1992, LECOMTE 1997). This paper studies the causes of early embryo abortion and suggests some ways for embryo rescue when *P. polyanthus* is the female parent. For the histological observations, our objectives were : (i) to understand causes of embryo abortion and (ii) to overcome barriers between the two *Phaseolus* species.

MATERIALS AND METHODS

Two genotypes per species were selected for their good adaptation (flower production) in our experimental conditions and higher potentialities for pod setting : G 21245 wild form (WLD) and NI 637 cultivated form (CV) in *P. vulgaris*, NI 1015 (CV) and G 35348 (CV) in *P. polyanthus* (GEERTS 2001). Plants were grown in a controlled chamber : 24°C/20°C day/night temperature, 75% relative humidity, 580 µmol m^{-2} s^{-1} light intensity (measurements at 60 cm from 400 watt

Grolux lamps) and 11 hours 30 min. day-length. Emasculation took place one day before anthesis. For each cross, five pods of maternal genotypes (*P. polyanthus* or *P. vulgaris*), containing three or seven seeds respectively, were inspected daily from 2 to 6 DAP. In order to confirm pollen germination, stigmatic surfaces were removed 2 DAP and were immediately incubated in aniline blue solution (0.01‰ in 0.1 M phosphate buffer, pH 9) for 10 min (RUZIN 1999). Under UV excitation, yellow fluorescence indicates pollen germination. Seeds from parental genotypes and from interspecific crosses were freshly harvested and nicked with a scalpel to facilitate penetration of fixing and embedding solutions. Samples were fixed in 1.2% glutaraldehyde in 0.3 M phosphate buffer for 48 h at 4°C, rinsed in phosphate buffer, dehydrated in a graded ethanol series and embedded in Technovit 7100 resin. Two µm-thick sections were cut on a Zeiss HM 360 microtome fitted with a tungsten-carbide knife. They were stained with a toluidine blue O procedure adapted from GUTMANN (1995). Histological observations were made from one-day before anthesis to 15-DAP (GEERTS 2001).

The potential of *in vitro* culture techniques to rescue immature embryos (SHARMA *et al.* 1996) was investigated. Our aim was twofold : (i) rescue of 2-4 days old embryos and (ii) development of *in vitro* culture techniques adapted to *P. vulgaris* and *P. polyanthus*. Materials included one genotype of *P. vulgaris* (NI 637) and two genotypes of *P. polyanthus* (NI 1015 and G 35348). Two major problems occur with the culture of very immature embryos : (i) the complexity of the required medium with very small embryos and (ii) the difficulty to extract very small embryos without damaging the suspensor. To reduce suspensor damage, we opted for a protected embryo culture, *in ovulo* or pod culture, during a limited time (5 to 10 days) before extracting the embryo at a stage when the role of the suspensor is much less important, that is to say the late heart-shaped or cotyledonary stage. Such an evolution was particularly observed with pods cultivated 4 DAP. Some embryos at globular developmental stage evolved to the cotyledonary stage. Therefore, pod culture techniques were chosen, in our case, with a view to rescue 2 day-old embryos and to study the impact of the osmolality adjustment in the media. The *in vitro* techniques involved three parameters likely to influence the success rate : (i) genotype, (ii) pod age (days of duration on the mother plant) before its *in vitro* culture, and (iii) osmolality evolution, using discontinuous values (with solid medium) or constant values (with liquid medium) (TOUSSAINT *et al.* 2002).

Experimentations conducted during several years (MERGEAI *et al.* 1997, LECOMTE 1997, GEERTS *et al.* 1999, GEERTS 2001, TOUSSAINT *et al.* 2002), allowed us to develop a step by step procedure of *in vitro* culture to rescue young embryos. The following procedure, described by GEERTS *et al.* (2001), was used : (i) *Phaseolus* pods were collected from 2 to 5 DAP and cultivated on solid or liquid medium (PHILLIPS *et al.* 1982, modified medium at 580, 450 and 350 mosm) ; (ii) after one week, embryos were extracted and cultivated on solid medium (PHILLIPS *et al.* 1982, modified medium at 350 mosm) for maturation during 2 weeks ; (iii) then, embryos were placed on dehydration medium (HU & ZANETTINI 1995, modified) during 2 weeks ; (iv) next, embryos were transferred in tubes for germination and rooting in presence of IAA (MERGEAI *et al.* 1997, modified medium) during 1 week ; (v) at the final step, after a period of *in vitro* growth of about 2 weeks (MERGEAI *et al.* 1997, modified medium), plantlets were transplanted in Jiffy pots for acclimatisation. Eight weeks later, plantlets were regenerated.

According to pod age, the osmotic pressure of culture media should evolve. When using solid media, this evolution is obtained by steps ; when using liquid media, the same evolution can be obtained in a continuous way. We designed, for liquid medium, a system enabling a continuous evolution of the osmotic pressure according to pod age (GEERTS *et al.* 2000). In a first step, pods were supported on glass beads in a Petri dish containing a medium with an osmolality adapted to preglobular embryos. The Petri dish was connected to a bottle decanter containing another medium with an osmolality adapted to the cotyledonary developmental stage. During the first 5 days, daily solutions were dripped from the bottle decanter to the Petri dish, enabling a constant evolution of the culture medium osmolality.

RESULTS AND DISCUSSION

Development of the parental *P. polyanthus* embryo showed the same features as those observed in *Phaseolus* embryony (YEUNG & CLUTTER 1978). Although development of the different tissues is identical between *P. vulgaris* and *P. polyanthus*, it is interesting to compare their embryogenesis. According to LECOMTE *et al.* (1998), the size of the suspensor is larger 4 DAP in *P. polyanthus* (70 µm) than in *P. vulgaris* (50 µm). Considering the role of the suspensor in the young embryo nutrition, this difference in size could

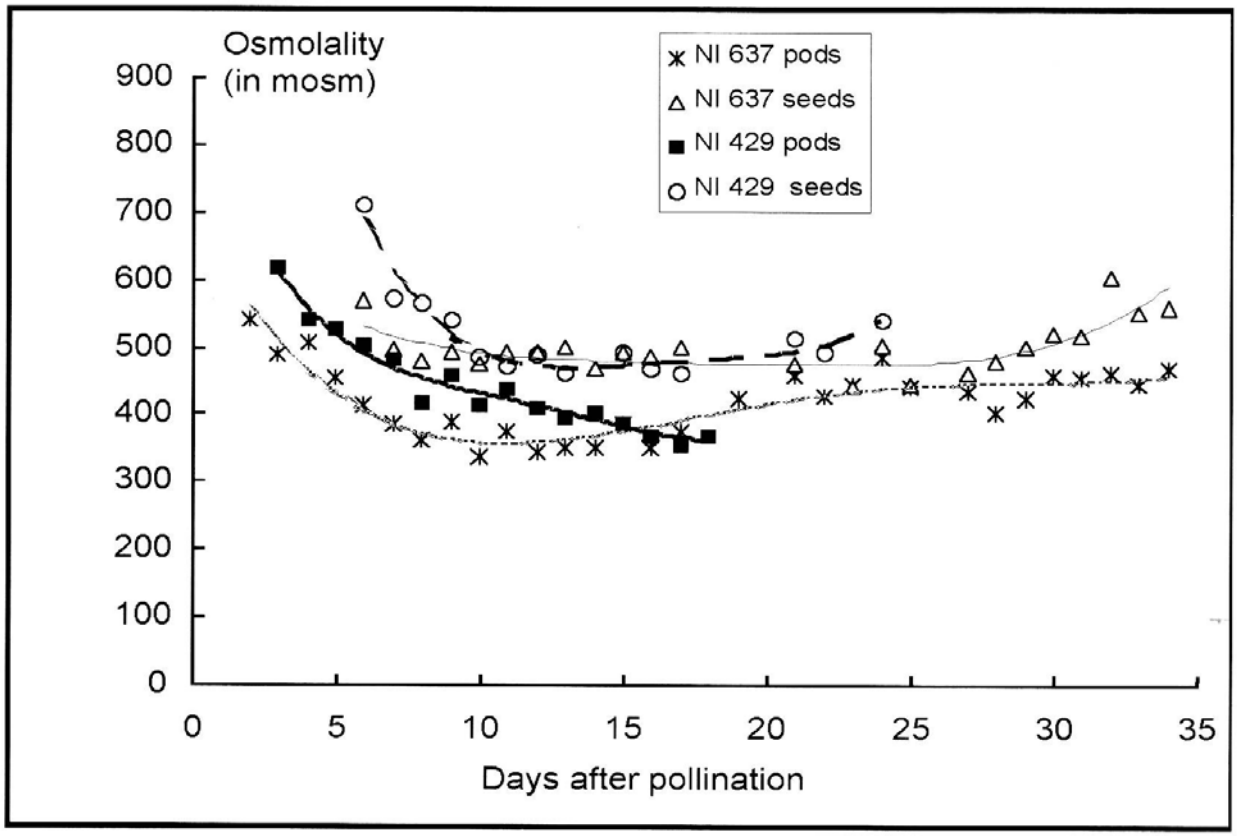

FIG. 1. — Evolution of osmolality in pods and seeds of two species : *P. vulgaris* (NI 637) and *P. polyanthus* (NI 429). Means of three repetitions.

explain abortion in the interspecific hybrids, particularly when *P. polyanthus* is used as female parent. Suspensor size of the hybrid embryo might not be adapted to the maternal environment, reducing consequently the embryo nutrition. Another observation supports this hypothesis. Embryo development is slower in *P. polyanthus* than in *P. vulgaris*. Five DAP, cotyledons are initiated in *P. vulgaris*, embryos being at the early heart-shaped stage, while *P. polyanthus* embryos are still at the globular stage.

Our histological examination could point out five major causes of seed abortion in *P. vulgaris* × *P. polyanthus* reciprocal crosses : (i) poor embryo development, (ii) limited endosperm division, (iii) endothelium proliferation, (iv) nucellus degeneration, (v) hypertrophied vascular elements. This was illustrated by the histological study of GEERTS *et al.* (2002).

In *P. vulgaris* × *P. polyanthus* crosses, early abortion is linked (i) with endothelium proliferation when *P. vulgaris* is used as female, and (ii) with low endosperm development when *P. polyanthus* is the female parent. Later (6 and 7 DAP), hybrid embryo abortion is mainly related with the abnormal development of the suspensor, which is detached from the growing embryo, when *P. vulgaris* is the female parent. In reciprocal crosses, late hybrid embryo abortion is mainly related with the degeneration of the nucellus and vascular tissues. This observation suggests an early reduction in nutrient transport from maternal tissue to the embryo sac.

Fig. 1 shows the evolution of osmolality in pods and seeds of the common bean and *P. polyanthus*. There is a gradient between seed and pod, as reported in the literature (YEUNG & BROWN 1982, CHAMBERLIN *et al.* 1993, GEERTS *et al.* 2000). In particular, GEERTS *et al.* (2000) indicated an osmolality higher in seeds than in pods but lower in seeds than in embryos. Modification of osmolality values occurred at two different

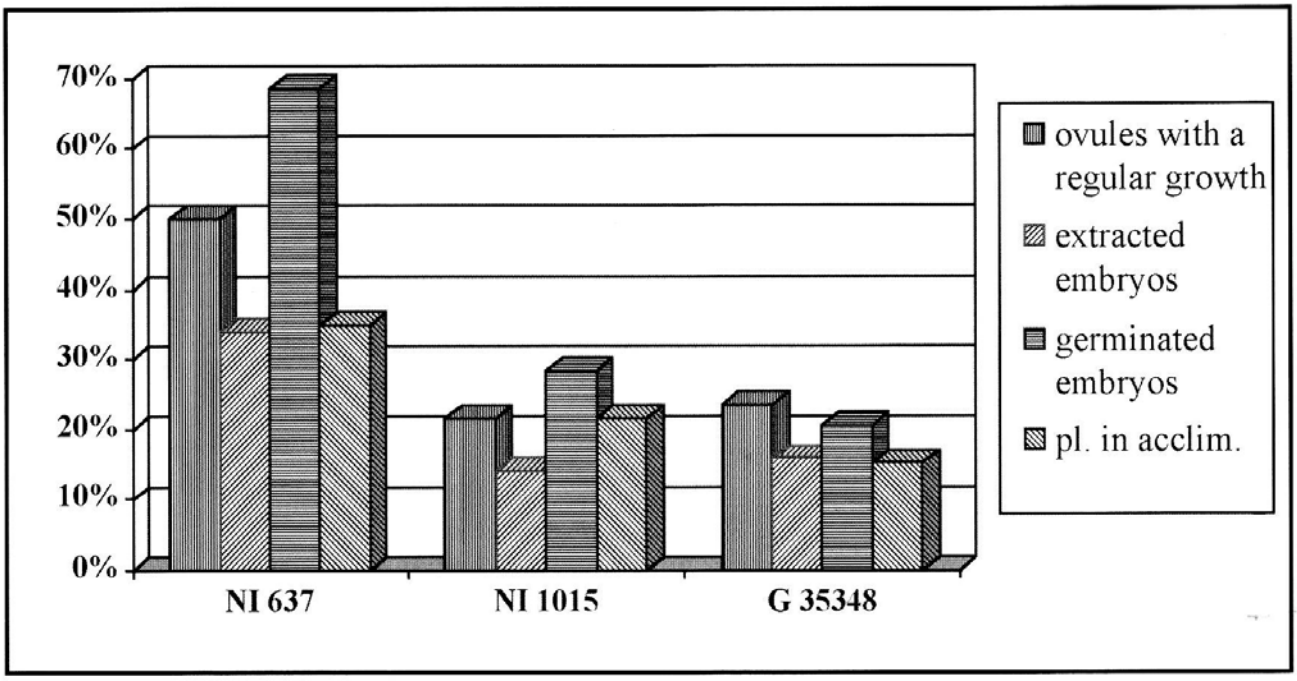

FIG. 2. — Influence of the genotype on rates of ovule growth, extracted embryos, germinated embryos and plantlets in acclimatisation (7 days of *in vitro* pod culture).

periods : (i) 22 DAP, corresponding to dehydration of seeds, and (ii) immediately after pollination, up to 11 days, when embryos reached cotyledonary stage. On the basis of these observations, we concluded that an osmotic gradient between pod, seed and embryo, should be maintained to ensure embryo development.

Figs. 2 and 3, and Table 1 summarise the influence of the three different parameters studied in *in vitro* culture. Fig. 2 shows the influence of the genotypes on rates of ovule growth, extracted embryos, germinated embryos and plantlets in acclimatisation, after seven days of *in vitro* pod culture. Frequency of ovules with a regular growth was higher in *P. vulgaris* than in *P. polyanthus*, and the rate of embryo extraction was twice higher in *P. vulgaris* than in *P. polyanthus*. Such results may be related with a delay of embryos evolution in *P. polyanthus*. Indeed, extracted embryos of *P. vulgaris* reached heart-shaped or cotyledonary stage while extracted embryos of *P. polyanthus* were hardly developed. Germination of extracted embryos was higher in *P. vulgaris* than in *P. polyanthus*, and the same trend was observed with a number of plants in acclimatisation. Table 1 illustrates the influence of pod age in *P. polyanthus* before its *in vitro* culture. No significant difference was observed between the three levels of pod age, although we noticed, as expected, a variation in the proportion of embryo developmental stages. The number of germinated embryos and plantlets in acclimatisation was rather constant. The influence of the pod culture techniques on the number of extracted and germinated embryos on the one hand, and of plantlets in acclimatisation on the other hand is shown in Fig. 3. Concerning *P. vulgaris,* no significant difference between the two media, solid and liquid, was observed with extracted or germinated embryos. On the other hand, a higher number of plantlets in acclimatisation were noticed with pod culture on liquid medium. Concerning *P. polyanthus,* the most significant results were the higher percentage of germinated embryos and plantlets in acclimatisation when pods were cultivated on solid medium, a behaviour different from *P. vulgaris* (TOUSSAINT *et al.* 2002).

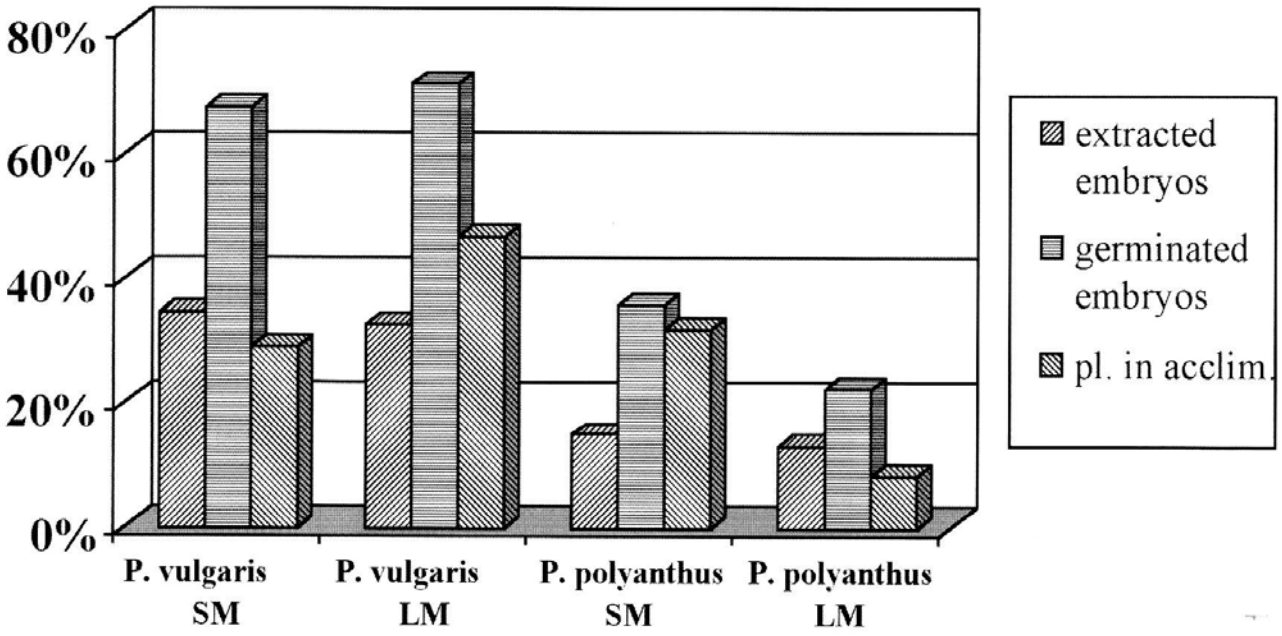

FIG. 3. — Results of pod culture techniques : importance of extracted and germinated embryos, and of plantlets in acclimatisation. Pod culture media : SM, solid medium ; LM, liquid medium.

TABLE 1

Influence of pod age in Phaseolus polyanthus *before its* in vitro *culture on the proportion of obtained embryos at different developmental stages*

Embryo stages	3 days	4 days	5 days
Globular	47.0 %	30.3 %	15.8 %
Young heart-shaped	45.1 %	39.4 %	39.5 %
Heart-shaped	7.9 %	30.3 %	42.1 %
Cotyledonar	0.0 %	0.0 %	2.6 %

CONCLUSIONS

As a conclusion, our investigations have pointed out several abnormalities during hybrid embryo development depending to a great extent on the compatibility between the genotypes used as parent. The development of embryo in *P. polyanthus* (♀) × *P. vulgaris* crosses was very poor. Our results show that early nutritional barriers are related with a deficient endosperm tissue development, while in reciprocal crosses, endothelium proliferation and in some extent, hypertrophy of the vascular elements can be considered as the main causes of early embryo abortion.

Water relation and osmolality conditions are important for developing a efficient *in vitro* culture technique. In particular, we showed (i) the necessity to adopt osmolality conditions close to those observed *in vivo* (within pod) during the early embryo development, and (ii) the key role of a dehydration medium between maturation, germination and rooting of embryos, which reflects the natural process of seed dehydration. On the basis of these modifications we could obtain, for the first time, fertile plants of *P. vulgaris* and *P. polyanthus* from pods harvested 2 to 4 DAP.

However, progress is still expected on several points. First, conditions of sterilisation, particu-

larly during *in vitro* pod culture, need to be improved. Indeed, numerous pods were lost because of bacterial or fungal contaminants. Second, better water relation conditions and the hormonal balance fitted to the various steps of our *in vitro* sequence should be identified. Finally, the techniques tested on the two *Phaseolus* species should be adapted to the interspecific hybrids *P. polyanthus* (♀) × *P. vulgaris*. This is essential to breed and develop *P. vulgaris* varieties combining the agronomical advantages of the common bean with the useful traits from the donor parent, *P. polyanthus*.

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