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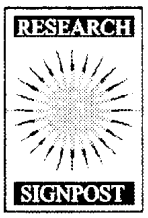

Recent Res. Devel. Genet. Breeding, 1(2004): 349-364 ISBN: 81-7736-218-6

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Interspecific hybridization with *Phaseolus vulgaris* L. : Embryo development and its genetics

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Abstract

Interspecific hybridization between Phaseolus vulgaris, the recurrent species, and the two donor species, P. coccineus or P. polyanthus, both used as female parent, is likely to bring useful agronomical traits poorly or not expressed in the primary gene pool of the common bean. As the crosses P. coccineus (♀) or P. polyanthus (♀) x P. vulgaris produce a very high rate of embryo abortion at an early developmental stage preceeding cotyledon initiation, progress in the crossing programme will depend upon the understanding of embryogenesis and post-zygotic barriers in the hybrids and the identification of genes

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implicated in the embryo development of Phaseolus. Past and currents results of interspecific hybridization, histology of embryo development and preliminary screening of candidate genes in embryogenesis are synthetized, a specific objective being to refine in vitro embryo culture on the basis of these investigations.

Introduction

Within the genus *Phaseolus*, the common bean, *Phaseolus vulgaris* L., is the most important species widely distributed in the world and occupies more than 90% of production areas sown to *Phaseolus* species [6]. It is cultivated primarily for its green pods, green shelled seeds, and dry seeds. Its dry seeds are a major component in the diet of human populations in Latin America, and in Central and East Africa. They usefully complement cereal seeds in the nutritional value of daily diet, mainly because of their protein and amino acid contents. *Phaseolus vulgaris* is adapted to several cropping systems, particularly in association with other food crops like maize, sorghum or pearl millet [57]. In the tropical regions, the common bean is characterized by low and instable seed yield. This is mainly due to the susceptibility of this crop to numerous pests and diseases : more than 200 pathogens have been reported attacking beans, some of them causing considerable economic losses [30, 57]. Other constraints limiting the yield is the lack of improved varieties tolerant to abiotic stress (poor soil, high temperature, drought, etc.). Agronomists and breeders have not found sufficient genetic variability within the primary gene pool of *P. vulgaris* [2, 9]. However, the alien gene pools offer very good breeding potentialities [2, 15, 17]. This is particularly the case of the two species *P. coccineus* L. and *P. polyanthus* Greenm., which show interesting traits not, or only poorly, expressed in the primary gene pool of *P. vulgaris*, such as resistance to some diseases (Ascochyta leaf spot, Bean Golden Yellow Mosaic Virus), cold tolerance, long multi-noded inflorescences, and thick stems [2, 10, 51]. To overcome biotic and abiotic constraints, interspecific hybridizations have been attempted between *P. vulgaris* and these two species, with the aim to introgress desired traits to the common bean. In these crosses, the use of *P. vulgaris* as female parent increases the percent of succeeded hybrids but, unfortunately, the presence of *P. vulgaris* cytoplasm provokes a quick reversal to the recurrent species, at the expense of the donor species [2]. Reciprocal crosses, a condition favouring the introgression of desired genes to the common bean, avoid the reversal process showed above, but are more difficult to obtain. Indeed, theses crosses lead to abortion of immature embryos, usually at the globular or early heart-shaped developmental stages, with most embryos aborting 3-6 days after pollination. If *in vitro* embryoculture is useful to rescue embryos at the cotyledonary stage, this method is less efficient when embryos abort at an earlier developmental

stage [37, 42]. Disruption of major genes involved in embryogenesis can cause the degeneration of interspecific embryos. Transcripts of these genes can be localized in the embryo proper, in endosperm or in maternal tissues around the embryo [47].

Objective of our research is to study mechanism of *Phaseolus* embryogenesis at the histological and genetical levels, with a view to increase the number of interspecific hybrids, involving *P. coccineus* or *P. polyanthus* cytoplasm. First, we summarize briefly the *Phaseolus* phylogeny; thereafter we develop our methodology to achieve our objectives, with some recent results obtained in interspecific crosses, histology and genetics of embryogenesis; and we terminate by prospects for the use of interspecific hybridization in common bean.

A brief summary of *Phaseolus* phylogeny

The genus *Phaseolus* is Neotropical in origin and may contain 50-60 species, pending additional germplasm explorations [16]. Understanding the relationships between these species is important to take benefit of increased variability in bean breeding and guide a crossing programme at interspecific level. Recent phylogenetic studies including both wild and domesticated *Phaseolus* species and using morphological, biochemical and molecular data have confirmed that the genus is monophyletic [15]. Sub-clades may exist at the sub-generic level [5, 18]. One lineage includes *P. vulgaris* while another includes *P. lunatus* [21, 22, 40]. The three species *P. vulgaris*, *P. coccineus* and *P. polyanthus* belong to the same evolutionary branch [52,53]. Each of the three taxa contains wild and domesticated forms at the intraspecific level, representing the primary gene pool. Each taxum can be considered as a secondary gene pool for the other two taxa [2]. The gene pools of *P.vulgaris*, *P.coccineus* and *P.polyanthus*, as well as of other *Phaseolus* taxa, are maintained in the world base germplasm bank of the "Centro Internacional de Agricultura Tropical", CIAT, Cali, Colombia.

To overcome major production constraints of *P. vulgaris* cultivars, interspecific hybridizations with the two closely related species, *P. coccineus* and *P. polyanthus*, have been undertaken in breeding programmes. From 1940 to 1985, *P. vulgaris* and *P. coccineus* were frequently inter-crossed. It was observed however from reciprocal crosses that segregants naturally reverted to the cytoplasm of the maternal parent [3]. Major genes have established a barrier between these two species, and chromosome pairing is not perfect but depends upon the parental genotypes.On the basis of results obtained in a large crossing programme [2, 4], *P. polyanthus* crosses more easily with *P. vulgaris* and, even more, with *P. coccineus*, compared to the interspecific hybridizations between *P. vulgaris* and *P. coccineus*. *P. polyanthus* belongs to the *P. vulgaris* clade, but

its nuclear genome has been introgressed with *P. coccineus* genes. The very close proximity between *P. polyanthus* and *P. vulgaris* justifies the priority given to the crosses between these two species. As we mentioned it previously, the best combinations should involve *P. polyanthus* cytoplasm in order to increase rates of introgression of useful genes from the donor species. The major reproductive barrier to interspecific hybridizations among the genus *Phaseolus* occurs at post-zygotic level, especially during early embryo development [3]. When maintained *in vivo*, embryos resulting from *P. polyanthus* (female) x *P. vulgaris* crosses develop poorly despite the close phylogenetic relationship of these species. The causal agents of these barriers should be investigated through histological and genetic studies of embryogenesis.

Interspecific hybridization

Materials and methods

In order to achieve our general objective, i.e. understanding *Phaseolus* embryogenesis, a preliminary step is to choose genotypes having different combining abilities among the parental species, i.e. *P. vulgaris*, *P. coccineus* and *P. polyanthus*. Such genotypes should preferentially have a good flowering behaviour under our experimental conditions and interesting agronomical values, in terms of seed productivity and adaptation to biotic and abiotic constraints of the tropical regions. Three genotypes of *P. vulgaris* (NI 622, NI 637, and G 21245), three genotypes of *P. coccineus* (NI 16, NI 889, NI 1111) and three genotypes of *P. polyanthus* (NI 1015, G 35348, NI 1123) were selected in setting up our interspecific crossing programme. Their status and origin are indicated in table 1. Seeds were skinned, humidified and pre-germinated in Petri dishes to allow a rapid germination, before sowing in a substrate mix (Klasmann 4 special number 26 : 80% peat, Rhin's sand 5% and 5.5-6 g of organic fertilizer). The plants were grown in a greenhouse from September 2002 to April 2003, at approximately 21°C/16,5°C (day and night) and 80% relative humidity. The plants were watered every day and a nutritive solution [10] was applied every month from one month after sowing.

As crosses involving *P. vulgaris* as maternal parent are relatively easy to obtain and are mainly carried out as a control, we have decided to concentrate our efforts to the reciprocal crosses *P. coccineus* (♀) x *P. vulgaris* and *P. polyanthus* (♀) x *P. vulgaris*. Flowers of maternal genotypes were emasculated one day before opening. After removing carefully the standard, the wings and the keel, the stamens were eliminated with forceps and the stigmatic surface was cleaned with a fine brush. To prevent cross contamination with foreign pollen, the forceps and the brush were dipped in ethanol before and after each emasculation. Cross-pollination was carried out immediately after emasculation or one day later with *P. vulgaris* pollen. The stigmatic surface of the female

Table 1. Parental genotypes used in the crossing programme

Species	Number	Status	Origin
P. vulgaris L.	NI 637	Cultivar	Brazil
	NI 622	Wild	Peru
	G 21245	Wild	Peru
P. coccineus L.	NI 16	Cultivar	Rwanda
	NI 889	Wild	Mexico
	NI 1111	Wild	Guatemala
P. polyanthus Greenm.	NI 1015	Cultivar	Guatemala
	G 35348	Cultivar	Mexico
	NI 1123	Wild	Guatemala

parents was covered with the male flower keel containing dehiscent anthers. That avoid external contamination and preserve humidity necessary for successful pollen germination. To verify hybridity of plants resulting from the crossing programme, genomic DNA was extracted from the parental genotypes and the presumed hybrids and submitted to a PCR reaction with SSR primers (in our case, we use primers BM 141 obtained from CIAT).

DNA isolation

Young and green leaves (100 mg) of *Phaseolus* parental genotypes and plants obtained after hybridizations were used for genomic DNA extraction. The samples were ground to a fine powder under liquid nitrogen with a mortar and pestle, and transferred to an eppendorf tube. After the transfer, 750 µL of warm (65°C) CTAB extraction buffer (250 mM Trisma Base, 50 mM EDTA, 3% CTAB, 2.1 M NaCl, 0.2% Mercaptoethanol) was added and the mixture was incubated for 20 min at 65°C in shaking water bath. Following incubation, 750 µL of chloroform/isoamylalcohol (24:1) was added, and the extract was thoroughly mixed and centrifuged for 15 min at 11,000g at 4°C. The aqueous phase was carefully transferred to a new tube. DNA was precipitated by adding an equal volume of isopropanol and incubation of the mixture for 30 min at –20°C. After centrifugation for 5 min at 8,000g at 4°C, the pellet was washed with 70% chilled ethanol, dried at room temperature for 20 min and dissolved in 70 µL of TE (10 mM Trisma Base, 1 mM EDTA) by a 10 min shaking water bath incubation at 60°C. To remove RNA traces, 5 µL of RNAse A (10 mg/mL) was added into DNA samples, followed by incubation at 37°C for 30 min in non-shaking water bath and centrifugation for 3 min at 8,000g at 4°C. The supernatant containing the DNA was transferred to a new tube and stored at 4°C.

PCR methods

The PCR reaction was performed using 20 ng of gDNA as template, 0.1 µM of each forward and reverse SSR primer pairs (BM 141 from CIAT, synthetised by Eurogentec), 2 mM $MgCl_2$, 0.25 mM each dNTP (Amersham Pharmacia

biotech), 1X PCR buffer and 1.5 units of *Taq* DNA polymerase (Amersham Pharmacia biotech) in a 20 µL final volume. PCR was carried out in thermal cycler (Peltier Thermal Cycler 200, MJ Research) with the following profile: 3 min hold at 94°C and 35 cycles of 15 sec denaturation at 94°C, 15 sec annealing at 55°C, 15 sec extension at 72°C, followed by a final extension at 72°C for 5 min. One volume of a denaturing solution (10 M NAOH, 95% Formamid, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol, in 10 mL of steril water) was added on the amplification products and the samples were heat denaturated at 90°C for 5 min. PCR products were separated on 6% denaturing polyacrilamide gels for 1 h 30 min, after a pre-run of the gels for 1 h.

Results of the crossing programme

Table 2 indicates the results from interspecific hybridizations, using only *P. vulgaris* genotypes as male parent. In our experimental conditions, the cotyledonary developmental stage of the embryos is usually obtained 14 or more than 14 days after pollination (DAP). Before 14 days, embryos are at the globular or heart-shaped developmental stages. The very short and not synchronized

Table 2. Percent of pod setting in interspecific crosses at different time intervals after pollination

Interspecific combinations	Number of cross-pollination	Time intervals after pollination				
		3-5 days	6-8 days	9-13 days	≥ 14 days	Mature pods
PP (♀) x PV						
G 35348 x NI 637	165	67.3	12.1	08.5	12.1	00.0
G 35348 x G 21245	176	72.2	10.2	07.9	09.1	00.6
G 35348 x NI 622	20	50.0	15.0	35.0	00.0	00.0
NI 1015 x NI 637	159	54.7	22.7	09.4	13.2	00.0
NI 1015 x G 21245	50	50.0	24.0	14.0	12.0	00.0
NI 1123 x NI 637	21	47.6	33.3	19.1	00.0	00.0
NI 1123 x G 21245	14	57.2	21.4	21.4	00.0	00.0
NI 1123 x NI 622	10	60.0	20.0	10.0	10.0	00.0
PC (♀) x PV						
NI 889 x NI 637	25	48.0	20.0	32.0	00.0	00.0
NI 889 x G 21245	34	44.1	17.7	11.8	23.5	02.9
NI 16 x NI 637	161	70.2	17.4	08.1	04.3	00.0
NI 16 x G 21245	103	66.0	18.5	10.7	04.8	00.0
NI 16 x NI 622	10	50.0	50.0	00.0	00.0	00.0
NI 1111 x NI 637	15	53.3	40.0	00.0	06.7	00.0
NI 1111 x G 21245	20	65.0	20.0	10.0	00.0	05.0
NI 1111 x NI 622	18	50.0	27.8	16.7	05.5	00.0

PV = *P. vulgaris*, **PP** = *P. polyanthus*, **PC** = *P. coccineus*

flowering period of some genotypes explains the low number of hybridizations carried out for some combinations. A total number of 615 crosses *P. polyanthus* (•) x *P. vulgaris* and 386 crosses *P. coccineus* (•) x *P. vulgaris* were carried out. Most pods (from 44.1 to 72.2 %) degenerated between 3 and 5 DAP, i.e. at the globular and early heart-shaped stages, as it was already observed in previous studies [4, 25, 37].

In the crosses *P. polyanthus* (•) x *P. vulgaris*, only one mature pod was obtained from the combination G 35348 x G 21245 on a total of 176 hybridizations, corresponding to a 0.6 % success rate. This rate is reduced to 0.2% on the basis of all the *P. polyanthus* x *P.vulgaris* combinations. Except the combination G 35348 x NI 622, the percent of pod setting 14 DAP ranged from 9.1 to 13.2%. In the crosses *P. coccineus* (•) x *P. vulgaris*, two mature pods were obtained : one from NI 889 x G 21245 on a total of 34 hybridizations (2.9% success rate) and the other from NI 1111 x G 21245 on a total of 20 hybridizations (5% success rate). These two pods represent 0.5 % success rate on the basis of all the combinations attempted between *P. coccineus* and *P. vulgaris*.

Usually, interspecific hybridizations using *P. vulgaris* as male parents lead to embryo abortion occurring at an early developmental stage [2, 10, 11, 36, 43, 44; 55, 58]. *In vitro* embryo culture was successful to regenerate plants, mainly when embryos reached the cotyledonary developmental stage [10, 11]. The low seed set in *Phaseolus* interspecific hybridization is attribued, partly to pre-zygotic barriers, partly to the slow development of embryo and endosperm at post-zygotic level [33, 59]. Considering the role of the suspensor in the young embryo nutrition, a study on *Phaseolus* embryogenesis [38] pointed out as a factor of embryo abortion suspensor abnormalities in interspecific hybridizations. According to various interspecific hybridization programmes [25, 37, 43, 46], parental genotypes influence also the severity of incompatibility barriers and hence the rate of embryo development. Such parental genotype influence can explain the unequal number of embryos surviving 14 DAP or reaching maturity, as showed by table 2.

Seeds of the three mature pods resulting from the following crosses: G 35348 x G 21245 (3 seeds), NI 889 x G 21245 (2 seeds) and NI 1111 x G 21245 (1 seed), were sown to check their viability and confirm the F_1 hybrid nature. The seed from the combination NI1111 x G21245 did not germinate. Seeds of the two other combinations germinated, but only one seed for each pod produced a plant with intermediate morphological characters between the two parental genotypes (particularly bractlet shape and stem and flower pigmentation). The two plants flowered and were fertile.

After a PCR reaction applied with the SSR primers on the genomic DNA extracted from young leaves in the parental genotypes and the presumed hybrids, PCR products were separated on a polyacrylamide gel. The profile of the analyzed samples confirms the morphological observations, with the two F_1

hybrid plants G 35348 x G 21245 and NI 889 x G 21245 showing intermediate phenotype which had the specific fragments of the male partner G 21245 [56].

Histology of embryogenesis

Method

For histological studies, objectives were twofold: to identify abnormalities in tissue development and to understand causes of embryo abortion in hybrids between the two *Phaseolus* species. For each cross, pods of maternal genotypes (*P. polyanthus, P. coccineus* and *P. vulgaris*) 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 MPO_4 buffer, pH 9) for 10 min [48]. Under UV excitation, yellow fluorescence indicates pollen germination. Seeds from parental genotypes and from interspecific crosses were freshly harvested and eventually nicked with a scalpel to facilitate penetration of fixing and embedding solutions. Samples were fixed on 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. Sections 2 µm thick were cut on a Zeiss HM 360 microtome fitted with a tungsten-carbide knife. They were stained with an adapted Toluidine Blue O procedure [31]. Histological observations were made from one day before anthesis to 15 DAP. Slides were observed with a Nikon photomicroscop (model Eclipse 800). Extracted embryos were observed under a binocular (Wild M3Z). Images were captured by a JVC 3 + CCD color camera of Sony (model KY-F58) and contrasted by the Archive Plus programme of Sony.

Results

Development of parental *Phaseolus* embryos showed the same features as those described in *Phaseolus* embryony [65]. It is however interesting to compare embryogenesis in *P. vulgaris* and *P. polyanthus*. According to a study made in our experimental conditions [38], the size of the suspensor is larger four DAP in *P. polyanthus* (70 µm) than in *P. vulgaris* (50 µm). Considering the nutritive and hormonal role of the suspensor in young embryo development, this difference in size could explain abortion in the interspecific hybrids *P. polyanthus* (•) x *P. vulgaris*. Suspensor size might not be adapted to the maternal environment, reducing consequently the embryo nutrition. In addition, 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. Figures 1 and 2 illustrate comparative embryogenesis between *P.vulgaris* and *P.polyanthus* in relation with suspensor size and embryo developmental rate. This marked difference in embryo development between the two parental *Phaseolus* species could explain the low rate of hybrid embryos observed in our crossing programme.

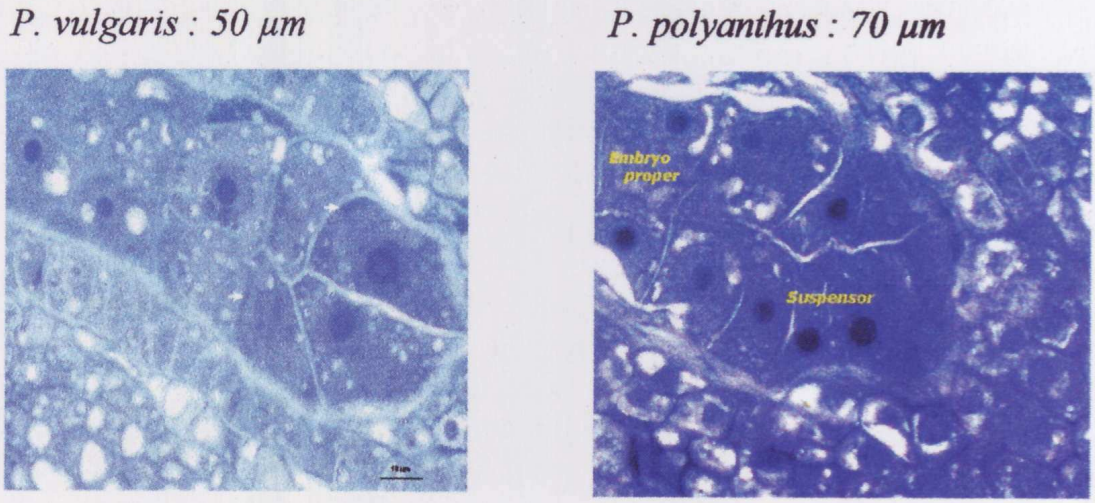

Figure 1. Comparative embryogenesis of *P. polyanthus* and *P. vulgaris*: suspensor size, 4 DAP.

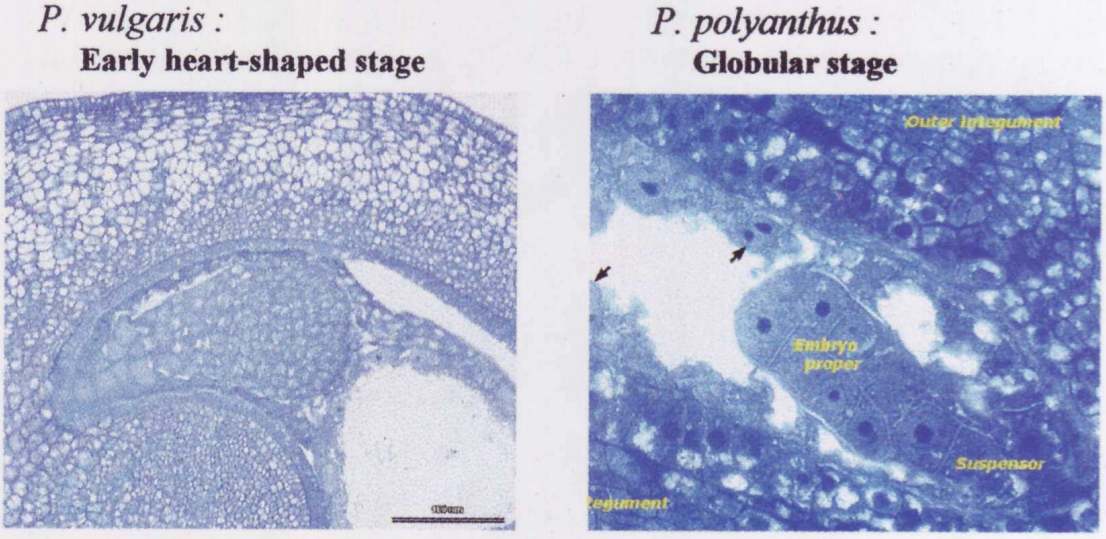

Figure 2. Embryo developmental stage 5 DAP in *P. vulgaris* and *P. polyanthus*.

The histological examination points out five major causes of seed abortion in the reciprocal crosses *P. vulgaris* x *P. polyanthus* : (i) poor development, (ii) limited endosperm division, (iii) endothelium proliferation, (iv) nucellus degeneration, (v) hypertrophied vascular elements. This was well illustrated by a recent histological study made in our experimental conditions [29].

In *P. vulgaris* x *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 the reciprocal cross, 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. Figures 3 and 4 illustrate some abnormalities observed in the embryo development of the cross: *P. polyanthus*, as female parent, by *P. vulgaris*.

Normal embryo development is also dependent upon the gradient of osmolality between embryo, seed and pod, evolving *in vivo* during the pod filling period. This has been reported in various investigations [12, 25, 27, 64]. In particular a study carried out under controlled conditions [27] indicated an osmolality higher in seeds than in pods but lower in seeds than in embryos.

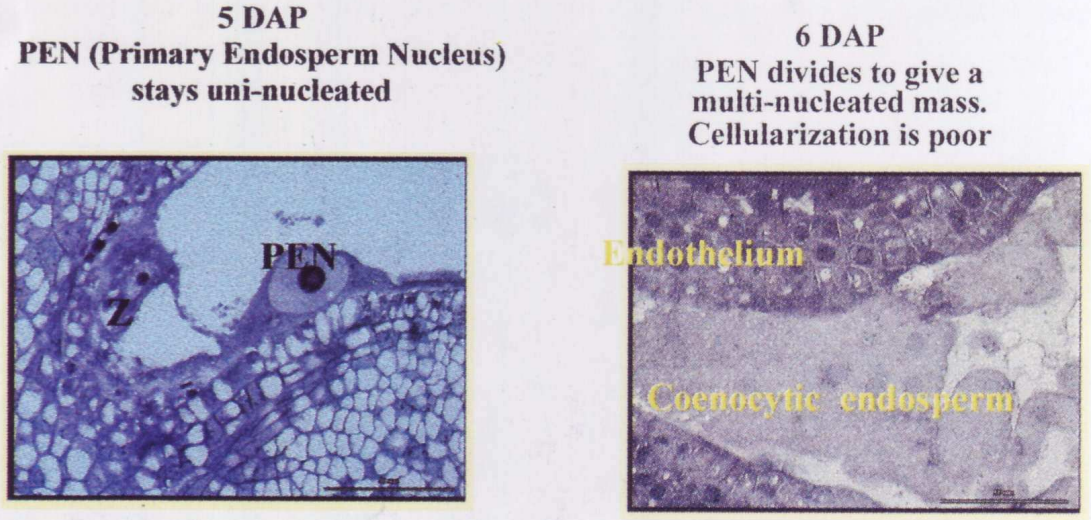

Figure 3. Limited endosperm division in *P.polyanthus* (♀) x *P.vulgaris*, 5 and 6 DAP

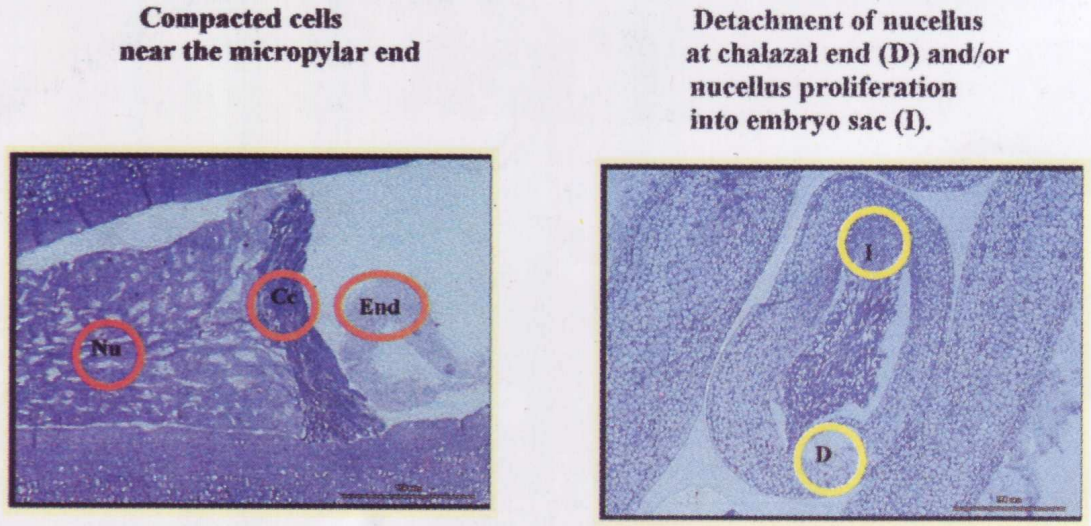

Figure 4. Nucellus degeneration in *P. polyanthus* (♀) x *P. vulgaris*, 5 DAP.

Modifications of osmolality values occurred at two different periods : (i) immediately after pollination, up to 11 days, when embryos reached cotyledonary stage, and (ii) 22 DAP, corresponding to dehydration of seeds. The osmotic gradient between pod, seed and embryo should be maintained *in vitro* to ensure proper *Phaseolus* embryo development.

Genetics of embryogenesis

In order to understand mechanisms of *Phaseolus* embryogenesis, it is essential to identify genes involved in this process and whose disruption can cause the abortion of interspecific embryos, and to study the spatial pattern of these genes inside the ovule. To tackle these problems, we have adopted two approaches. The first approach is the molecular analysis of model plant genes involved in embryogenesis with a view to identify *Phaseolus* homologous genes. Indeed, significant genes in the embryogenesis process of model plants such as *Arabidopsis thaliana* (L.) Heynh, *Zea mays* L., *Oryza sativa* L., etc. have been studied [7, 8, 19, 20, 24]. Such genes have already been sequenced and the effects of proteins resulting from their expression on the embryonic development as well as the consequency of possible disruption on the embryo survival are known. Among genes studied in model plant embryogenesis, MONOPTEROS, KEULE, TWN1 and HOMEOBOX have been isolated in *Arabidopsis* [1, 32, 41, 62]. The second approach is the isolation of specific genes implicated in *Phaseolus* embryogenesis using the "Differential Display" technique [39] on mRNA extracted from the self-pollinated ovules of various parental genotypes and from the degenerated ovules from interspecific hybridizations. The fragments so revealed will be isolated, cloned, sequenced and compared with other already isolated genes.

Among the genes involved in embryogenesis, HOMEOBOX genes are very often reported in both plant and animal species; homology of sequences is revealed between these genes belonging to distinct species. HOMEOBOX genes are regulator of transcription, either enhancer or inhibitor. Such genes control expression of other genes. They play a significant role in the development of living organisms, before and after embryogenesis [13, 34, 49, 50, 63]. From their structure and homology of sequences, homeodomain proteins characterizing these genes can be grouped in several classes : leucine zipper (HD-Zip), finger (PHD finger), GLABRA – 2 like (HD-GL2) from *Arabidopsis* and rice, Knotted 1-like (KNOX) from maize, etc. [35, 45, 50, 63]. In addition to HOMEOBOX genes, other genes play an important role in plant and animal embryogenesis : heat shock response or HSR, EMP2 or ZmHSBP1 and

ZmHSBP2 from maize, AtHSBP1 from *Arabidopsis*, OsHSBP2 from rice, MtHSBP1 from *Medicago*, GmHSBP1 from soya, etc. [23, 54].

In our investigations, a special attention was devoted to KNOX genes or Knotted – like of maize. The KNOX genes control the development and the shape of leaves by acting on apical meristems during the first stages of embryogenesis and also during the later vegetative and generative development stages. KNOX genes expression during the early periods of embryogenesis could be used as molecular markers for meristem initiation. This expression starts from the first developmental stages of the globular embryo and is present in the area where the apical meristem will develop precisely. To identify this gene family in *Phaseolus*, we made first an alignment of protein sequences of some KNOX genes in rice, maize, *Arabidopsis* and soya. This alignment can be obtained thanks to "emma" informatic programme of EMBnet. Degenerated primers can be elaborated from the conserved amino acids of the homeodomain of KNOX genes and taking on account the preferential codons for each *Phaseolus* amino acid. These primers were utilized to carry out PCR from genomic DNA of *Phaseolus* and rice (as control), as this gene family has already been identified in *Oryza sativa* and fragments were amplified in *Phaseolus* and rice. This gene family was revealed by PCR amplification on genomic DNA extracted from young leaves of *Phaseolus* and rice. A pair of primers designed on the basis of conserved amino acids region of the homeodomain (PELDQFM: 5'-CCN-GAR-YTN-GAY-CARTTY-ATG-3' and QINNWFI: 5'-CAA-TGA-CGC-TTA-CGT-TGG-TT-3') of knotted-like genes of model plants were synthesized. The reaction mixture contained 20 ng of gDNA as template, 0.6 µM of each primer pairs, 4 mM of $MgCl_2$, 0.3 mM each dNTP (Amersham Pharmacia biotech), 1X PCR buffer and 0.002 U/µL of *Taq* DNA polymerase (Amersham Pharmacia biotech) in a 20 µL final volume. PCR reaction was performed using the following profile: 94°C for 4 min, 40 cycles of (94°C for 1 min, 55°C for 1 min, 72°C for 2 min), 72°C for 5 min and 20°C for 5 min.

In our investigations, so far, we identified in the genus *Phaseolus* a second gene family, Lipid Transfer Protein, involved in model plant embryogenesis. This gene family was isolated using the following specific primers: GAGTTGTTTCCATGGCCACC (forward) and GAGTAGTTTTCAGTGCCTTC (reverse) [14], and 'Titan One Tube RT-PCR' kit for RT-PCR reaction applied on RNA extracted from *Phaseolus* genotypes young leaves. RNA was extracted using Invitrogen's TRIZOL Reagent and the protocol provided. The mixture 1

of RT-PCR reaction contained 0.2 mM of dNTPmix, 5 mM of DTT, 5 U of RNase inhibitor, 0.4 µM of each primer, 1 µg RNA in a total volume of 25 µL. Mixture 2 contained 1X of RT-PCR buffer, 0.04 U of Enzyme in a total volume of 25 µL. Mixture 2 was added to mixture 1 and RT-PCR reaction was carried out using the following profile: 50°C for 30 min, 94°C for 2 min, 35 cycles of (94°C for 30 sec, 45°C for 30 sec, 68°C for 45 sec), 68°C for 7 min and 20°C for 5 min.

PCR and RT-PCR products were separated on 1% of agarose gels for 90 min to 120 min, after addition of equal volume of a denaturing solution.

In a next step, the candidate genes so revealed will be sequenced and the sequences obtained will be used to produce nucleic probes. *In situ* hybridization with the studied genes will also be performed at different evolution stages of the ovules resulting from self pollinations and interspecific hybridizations; this will enable us to locate the spatial pattern of these genes inside the ovule.

Prospects

Considering the embryo abortion at the globular or early-heart stage in the crosses *P. coccineus/P. polyanthus* (as female) x *P. vulgaris*, investigations on *in vitro* embryo culture follow two main objectives : first to delay as far as possible the extraction of the embryo from pod and ovule, and second to adjust water relation and osmolality conditions to the gradients observed during the *in vivo* pod development and ripening. At this stage, a step by step procedure has been developed for the rescue of the globular embryos belonging to the three parental species : *P. vulgaris, P. coccineus* and *P. polyanthus* [25, 26, 28, 37, 42, 60, 61]. It remains now to adapt this technique with the interspecific hybrids involving *P. coccineus* or *P. polyanthus* cytoplasm. This is essential to breed and develop *P. vulgaris* varieties combining the agronomical advantages of the common bean with the useful traits from the two donor species, i.e. *P. coccineus* and *P. polyanthus*.

In the genetic improvement of a crop, a significant progress in breeding for higher seed yield, diseases and pest resistance and adaptation is very often linked with the possibility to valorize the whole genetic diversity available at intra- and interspecific levels. In the case of the common bean, an important food crop in the tropics, the development of new interspecific hybrids *P. coccineus* (♀) or *P. polyanthus* (♀) x *P. vulgaris* will offer the breeders a valuable breeding stock to overcome the numerous biotic and abiotic constraints observed in the various cropping systems of Latin America and Africa.

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