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Protoplast fusion technology for somatic hybridisation in *Phaseolus*

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The success of interspecific breeding between *Phaseolus vulgaris* L. (PV) and the two donor species *Phaseolus coccineus* L. (PC) or *Phaseolus polyanthus* Greenm. (PP) requires the utilization of the donor species as female parents. Although incompatibility barriers are post-zygotic, success in such F1 crosses is very limited due to early hybrid embryo abortion. Rescue techniques for globular or early heart-shaped embryos have been improved but hybrid plant regeneration remains very difficult. In this study we describe the use of protoplast fusion techniques within the genus *Phaseolus*, as an alternative to succeed crosses between PP or PC and PV. Large numbers of heterokaryons have been produced using different genotypes and procedures for fusion, based either on electro-fusion (750 or 1500 $V \cdot cm^{-1}$), or on the use of a chemical micro-method with polyethylene glycol (PEG 6000) as the fusing agent. Both divisions of heterokaryons and the formation of heterokaryon-derived microcalli were observed.
Keywords. Protoplast, fusion, *Phaseolus vulgaris*, *Phaseolus polyanthus, Phaseolus coccineus*.

La technologie de fusion de protoplastes comme outil pour l'hybridation somatique chez *Phaseolus*. Le succès d'un croisement interspécifique entre *Phaseolus vulgaris* L. (PV) et les deux espèces donneuses, *Phaseolus coccineus* L. (PC) ou *Phaseolus polyanthus* Greenm. (PP), nécessite l'utilisation de ces dernières en tant que parents femelles. Bien que les barrières d'incompatibilité soient post-zygotiques, le succès de tels croisements F1 est très limité en raison d'un avortement précoce de l'embryon. Les techniques de sauvetage d'embryon globulaire et cordiforme jeune ont été améliorées mais la régénération de plantes hybrides reste très difficile. Dans cette étude, nous décrivons l'utilisation de techniques de fusion de protoplastes au sein du genre *Phaseolus* comme une alternative au succès des croisements entre PP ou PC et PV. Un nombre élevé d'hétérocaryons a été produit en utilisant différents génotypes et différentes procédures de fusion, basées essentiellement sur l'électro-fusion (750 ou 1500 $V \cdot cm^{-1}$), ou sur l'utilisation d'une technique micro-chimique, le polyéthylène glycol (PEG 6000) étant l'agent de fusion. Tant la division des hétérocaryons que la formation de microcals dérivés de ces hétérocaryons ont été observées.
Mots-clés. Protoplaste, fusion, *Phaseolus vulgaris*, *Phaseolus polyanthus, Phaseolus coccineus*.

1. INTRODUCTION

Major production constraints of the common bean *Phaseolus vulgaris* L. (PV) in Latin America and Africa are *Ascochyta* leaf blight, Bean Golden Mosaic Virus (BGMV), and Bean Fly (Obando et al., 1990; Baudoin, 1992). Sources of resistance have been identified in secondary gene pools, especially in *Phaseolus coccineus* L. (PC) and *Phaseolus polyanthus* Greenm. (PP) (Baudoin, 1992). To succeed interspecific crosses between PV and PP or PC, Camarena et al. (1987) and Baudoin et al. (1992) underlined the importance of using PP or PC as female parent to avoid a quick reversal to the recurrent parent PV. However, when using PP or PC cytoplasm in interspecific crosses with PV, incompatibility barriers are expressed at the globular or early heart-shaped embryo stages (Geerts et al., 1999; 2002). Previously, Shii et al. (1982) and Kuboyama et al. (1991) demonstrated that these barriers are post-zygotic and not due to pre-fertilization events.

Therefore, investigations have been carried out to improve embryo rescue techniques. One of the most efficient techniques in *Phaseolus* was based on micropod

culture: investigations were initiated by Geerts et al. (2000; 2001) and improved by Schryer et al. (2005). Although those techniques are now in an advanced stage of development, success in hybridisation remains extremely low.

As Zambre et al. (2001) reported the regeneration of PP plants from callus, focus was made on the possibility to use protoplast fusion techniques and somatic hybridization to overcome incompatibility barriers between maternal tissue of PV and hybrid embryo (Geerts et al., 2001).

Techniques for protoplast isolation and fusion are poorly studied within grain legumes (Ochatt et al., 2005; 2007), and no paper reports the use of protoplast fusion technology between PV and PP or PC. To initiate our research, the protocol described by Durieu et al. (2000) for intergeneric fusion of pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.) protoplasts was adopted. In a previous study, Ochatt et al. (2000) compared the effects of different enzymatic mixtures dissolved in various media on the efficiency of protoplast isolation and subsequent plant regeneration. Notably, they reported large differences between cellulases (Cellulase Fluka, Cellulase Onozuka RS or cellulase Onozuka YC) for the isolation of pea protoplasts and the favourable role of picloram in the regeneration medium.

In this study, we report the first results on protoplast fusion between PP or PC and PV using a protocol derived from that of Durieu et al. (2000).

2. MATERIALS AND METHODS

2.1. Plant material and growing conditions

From the *Phaseolineae* active collection held at Gembloux Agricultural University (Belgium), we selected one PP cultivar (NI1015), two PC cultivars (NI0016 and NI0229) and two PV cultivars (NI637 and NI638), according to their ability to grow *in vitro* (Lecomte, 1997).

Seeds were surface-sterilized in 12% calcium hypochlorite for ten minutes, and then immersed in 70% ethanol for 30 sec and rinsed three times in sterile de-ionised water. Seed scarification, humidification and pre-germination were carried out in sterile Petri dishes during 10 days.

Germinated seeds were first transferred into standard Bottles (Weck) containing 100 ml vermiculite and a standard Gamborg et al. (1968) half-strength medium solidified with 2 $g \cdot l^{-1}$ phytagel (Sigma) until lateral buds developed. Plantlets were then transferred into a new standard Bottle (Weck) onto a solidified MS medium (Murashige et al., 1962) containing 20 $g \cdot l^{-1}$ sucrose and 5 $g \cdot l^{-1}$ agar (Pastagar B).

Growing conditions were 24°C/21°C day/night temperatures with a 16 h photoperiod (Sylvania Gro Lux light, 54 $\mu E \cdot m^{-2} \cdot s^{-1}$).

2.2. Standard protoplasts isolation

Protoplasts of PC (NI0016 and NI0229) were isolated from green leaves. Fresh green leaves were more difficult to obtain with PV and PP genotypes when grown *in vitro*. Therefore, protoplasts were isolated from 10 day-old hypocotyl explants after pre-germination in Petri dishes for PV (NI637 and NI638) and PP (NI1015) accessions. Material was finely chopped and plasmolysed for 1 h in 10 cm^3 CPW medium (Frearson et al., 1973) with 10 mM $CaCl_2$, 13% mannitol and adjusted to pH 5.5 (CPW 13M). Tissues of all accessions (PV, PC, PP) were digested overnight on a continuous rotary shaker (60 $T \cdot min^{-1}$) with an enzyme mixture of 3% Macerozyme R10, 4% cellulase Onozuka RS, and 0.2% Pectolyase Y-23 (described as 3402RS by Ochatt et al., 2000). For PC accessions, we compared the use of cellulase Onozuka YC (described as 3402YC by Ochatt et al., 2000) versus cellulase Onozuka RS in the enzyme mixture. Onozuka YC was tested regarding the difference in source tissues (leaf explants versus hypocotyl).

2.3. Isolation of protoplasts for fusion

Isolation was carried out following Durieu et al. (2000) procedure. Briefly, protoplasts were sieved (40 μ for PV and PP and 50 μ for PC) and centrifuged successively at 35 g (5 min, 10°C) and 70 g (5 min, 10°C). Each pellet was resuspended in 200 mm^3 CPW13M. Pellets were mixed together and labelled with five drops (about 150 mm^3) of fluorescein diacetate (green, described as FDA) for PV accessions, while rhodamine B isothiocyanate (red, described as RBi) was used for PP and PC accessions. The use of both fluorochromes is described by Durieu et al. (2000). Stock solutions of fluorochromes were made from 5 mg for FDA or 30 mg for RBi per cm^3 acetone solution. Pellets were finally layered on top of 6 cm^3 of CPW solution containing 21% sucrose (CPW21S) and spun at 80 g (10 min, 10°C, maximum acceleration). Under UV light, protoplasts with FDA staining gave a yellow-green fluorescence allowing density and viability evaluation (Widholm, 1972), while those with RBi gave a red fluorescence (Durieu et al., 2000). Density is determined using a Bürker cell (Marienfeld, Germany). Optimum plating density is between 5 x 104 and 1 x 106, maximising wall regeneration and concomitant daughter cell

formation (Davey et al., 2005). Viability expressed as percentage is determined as the number of protoplasts that fluoresced yellow-green under UV light out of the total number of isolated protoplasts observed in the same microscopic field under normal light.

2.4. Protoplast fusion

Regarding the low density of protoplasts obtained for NI637 as PV accession (**Table 1**), electro-fusion could not be easily realized. Therefore, chemical fusion was conducted to perform protoplast fusion between NI637 with all PP and PC accessions. The micro-method described by Durieu et al. (2000) where PEG 6000 is the fusing agent was adopted.

For NI638 as PV accession, giving high protoplasts yield (**Table 1**), electro-fusion was tested with all PP and PC accessions. Electro-fusion was made following the method of Durieu et al. (2000) using 2 ml cuvettes of an Electro cell Manipulator ECM® 630 (BTX, California) with electrodes 1 mm apart. Three pulses at 750 $V \cdot cm^{-1}$ or 1500 $V \cdot cm^{-1}$ were delivered at 10 s intervals (capacitor of 75 μF, resistance of 50 Ω).

The efficiency of protoplast fusion with the two tested method was evaluated under UV light, as the fluorochromes are linked to different parental protoplasts, whereby heterokaryons can be observed and counted through their double fluorescence, green and red.

Table 1. The influence of genotype and enzyme mixture on *Phaseolus* protoplasts yield and their viability — *Influence du génotype et du mélange enzymatique sur le rendement en protoplastes de* Phaseolus *et leur viabilité.*

Genotype	**Enzyme mixture**	**Protoplast yield** ($10^3 \cdot ml^{-1}$)	**Viability** (%)
NI0016 (PC)	3402-YC	377	88.0 ± 4.1
	3402-RS	412	90.8 ± 4.3
NI0229 (PC)	3402-YC	886	91.8 ± 4.2
	3402-RS	760	89.0 ± 5.8
NI1015 (PP)	3402-RS	258	92.4 ± 5.2
NI638 (PV)	3402-RS	516	90.6 ± 5.8
NI637 (PV)	3402-RS	188	91.9 ± 4.7

PV = *Phaseolus vulgaris*; PC = *Phaseolus coccineus*;
PP = *Phaseolus polyanthus*.
Values are given with their standard error (± SE) — *Les valeurs sont données avec l'erreur standard (± SE).*
*Enzyme mixture is characterized by number 3402 for 3% Macerozyme R10, 4% cellulase Onozuka, and 0.2% Pectolyase Y-23 and the type of cellulase Onozuka used: RS or YC — *Le mélange enzymatique est caractérisé par le nombre 3402 signifiant 3 % de Macerozyme R10, 4 % de cellulase Onozuka, et 0,2 % de Pectolyase Y-23 et par le type de cellulase Onozuka utilisé : RS or YC.*

2.5. Culture

Protoplasts were cultured at 10^5 cm^{-3} on a medium based on KM (Kao et al., 1975) with 0.1 $mg \cdot l^{-1}$ 2,4-D, 0.2 $mg \cdot l^{-1}$ zeatin and 1 $mg \cdot l^{-1}$ NAA (described as KP). After one week a dilution was performed with the same medium and, as soon as the majority of cells had regenerated their wall, weekly dilutions (adding weekly 1 ml media per ml initial protoplast culture) were carried out with the culture medium containing 20 $g \cdot l^{-1}$ sucrose and 10 $g \cdot l^{-1}$ glucose.

3. RESULTS AND DISCUSSION

3.1. Protoplast isolation

Table 1 presents the results concerning the influence of genotypes and enzyme mixtures on protoplast yield and viability. A high dependency on genotype is observed whereas the influence of the tested enzyme mixture, containing either Cellulase Onozuka RS or YC, was not significant. To initiate protoplast isolation, we tested successfully enzyme mixture 3402 (Ochatt et al., 2000) as shown in **figure 1A**. Those results are largely supported by the literature (Davey et al., 2005) on protoplast culture pointing out that success in protoplast isolation is mostly genotype-dependent. Protoplast viability was also very high (**Table 1**) suggesting that CPW13M medium, characterised by the presence of high level of Calcium and Mannitol, is as well adapted to *Phaseolus* protoplasts as it is for pea protoplasts (Ochatt et al., 2000). Although our results allowed an optimal culture density of 10^5 protoplast per ml, some improvement can be achieved through the adjustment of various parameters, such as plasmolysis, enzyme concentration, the time of incubation and/or mannitol concentration aiming at a larger yield and coupled with an improved initial culture response (Davey et al., 2005).

3.2. Protoplast fusion

Both chemical and electrofusion techniques led us to form heterokaryons as shown in **figure 1**. Chemical fusion with protoplasts of PV genotype NI638 was more efficient than electrofusion with protoplasts of PV genotype NI637 (**Table 2**): the number of viable heterokaryons was above 10% in some cases and all combinations provided heterokaryons. However, the latter obtained by this method were not able to divide correctly compared to electro-fused protoplasts (**Table 2**). **Table 2** also shows that the use of a high voltage (1500 $V \cdot cm^{-1}$) was more effective to obtain heterokaryon-derived colonies. These heterokaryons could evolve to at least 10 cells microcallus within 25 days after fusion with some combinations, which underlines once a putative genotype dependence.

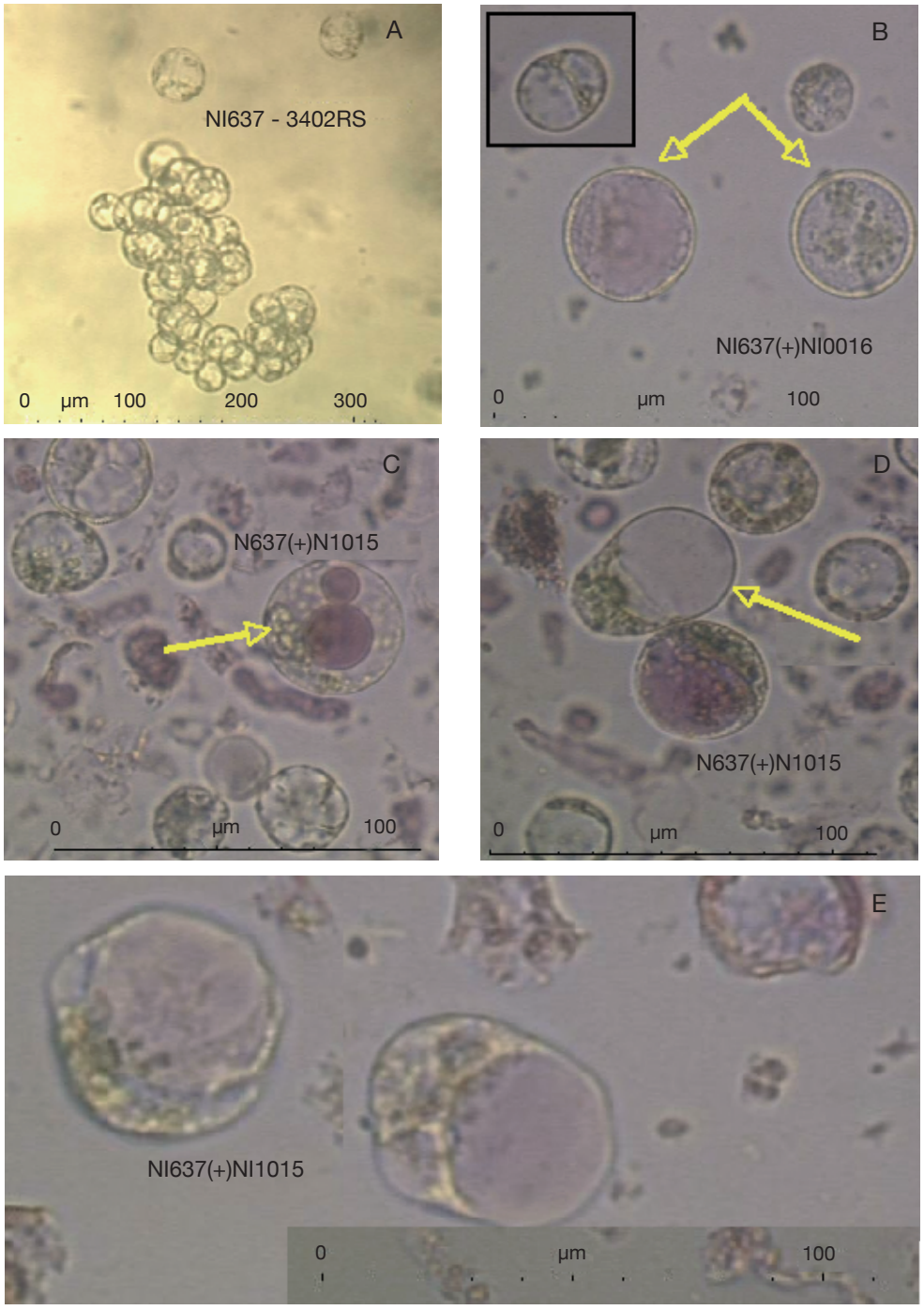

Figure 1. Protoplast fusion of *Phaseolus vulgaris* (NI637, NI638) and the related species *P. coccineus* (NI0016) and *P. polyanthus* (NI1015) — *Fusion de protoplastes de* Phaseolus vulgaris *(NI637, NI638) avec les espèces* P. coccineus *(NI0016) et* P. polyanthus *(NI1015) apparentées*.

A: freshly isolated NI637 protoplasts obtained with enzyme mixture 3402RS — *Protoplastes de NI637 nouvellement isolés obtenus avec le mélange enzymatique 3402RS* ; B: heterokaryons from a NI637 (+) NI0016 fusion showing dual labelling by the differential fluorochromes (arrowed); in medallion, a dividing heterokaryon-derived cell of the same combination of parental genotypes — *hétérokaryons d'une fusion NI637 (+) NI0016 montrant le marquage double par l'utilisation de fluorochromes différentiels (flèche) ; en médaillon, la division d'une cellule dérivée d'un hétérokaryon de la même combinaison de génotypes parentaux* ; C to E: results obtained following a NI637(+)NI1015 protoplast fusion — *résultats obtenus après la fusion de protoplastes NI637(+)NI1015* ; C: shows a multiple fusion (arrow) and several fusions of two protoplasts — *montre une fusion multiple (flèche) et différentes fusions de deux protoplastes ;* D: depicts three heterokaryons where one has regenerated a cell wall and entered mitosis (arrow) — *décrit trois hétérokaryons dont l'un qui a régénéré une membrane cellulaire entre en mitose (flèche)* and E: is a representative field after 10 days of culture with division of heterokaryon-derived cells — *est un champ représentatif de l'évolution après 10 jours de culture avec la division des cellules dérivées de l'hétérokaryon ;* A-E: were taken under transmission light — *ont été prises sous lumière de transmission*.

Table 2. Influence of genotype, enzyme mixture and fusion technique on the percentage of *Phaseolus* heterokaryons (observations made 5 days after fusion, mean data from a minimum of 200 protoplasts counted) and their subsequent proliferation to cell colonies and microcalli (observations made 25 days after fusion) — *Influence du génotype, du mélange enzymatique et de la technique de fusion sur le pourcentage d'hétérocaryons de* Phaseolus *(observations réalisées 5 jours après fusion, moyennes d'un minimum de 200 protoplastes comptés) et leur prolifération en colonies cellulaires et microcals (observations réalisées 25 jours après la fusion).*

Accessions			**Chemical**		**Electrofusion**			
PV	**PC**	**PP**	**PEG 6000**		**750 V·cm^{-1}**		**1500 V·cm^{-1}**	
			H	**M**	**H**	**M**	**H**	**M**
NI638	NI0016 -RS		5.5	5 to10				
NI638	NI0016 -YC		17.8	5 to10				
NI638	NI0229-RS		8.3	<5				
NI638	NI0229-YC		15.3	<5				
NI638		NI1015	9.5	<5				
NI637	NI0016 -RS				6.7	5-10	4.3	5 to10
NI637	NI0016 -YC				6.7	5-10	3.5	>10
NI637	NI0229-RS				1.5	5-10	5.8	>10
NI637	NI0229-YC				1.0	5-10	1.0	>10
NI637		NI1015			3.8	5-10	2.0	5 to10

PV = *Phaseolus vulgaris*; PC = *Phaseolus coccineus*; PP = *Phaseolus polyanthus*; RS and YC = type of cellulase Onozuka used during isolation — *type de cellulase Onozuka utilisée pendant l'isolation* ; H = percentage of heterocaryons observed 5 days after fusion — *pourcentage d'hétérocaryons observés 5 jours après la fusion* ; M = number of cells observed per microcallus 25 days after fusion — *nombre de cellules par microcals observés 25 jours après la fusion.*

NI637(+)NI0016-YC gives the most interesting microcalli in terms of viability and growth. Cytogenetics and flow cytometry studies (not shown) have attested that DNA content was more similar between NI637 and NI0016 than between other accessions, suggesting that such cytological studies could be used for a first screening of accessions. It is largely recognized that protoplast fusion between species having a significantly different nuclear DNA content is so far very difficult to obtain (Davey et al., 2005; Ochatt et al., 2007). Forthcoming experiments will look deeply into this aspect and will also address the sustained subsequent proliferation of the heterokaryon-derived tissues towards the ultimate regeneration of somatic hybrids between *Phaseolus* species.

4. CONCLUSIONS AND PERSPECTIVES

These first results using protoplast fusion technology for *Phaseolus* enlarge breeding perspectives for the improvement of common bean through interspecific hybridisation of a high interest. They describe a method allowing to screen rapidly and at an early stage genotypes showing potential ability to be fused through protoplasts and to generate microcalli.

The fusion technique still needs a number of adjustments to increase the viability of heterokaryons and their further evolution to microcalli. In this context, several factors such as the enzyme mixture, the fusion agents, the ratio between protoplasts belonging to each partner and the culture media could all have an influence on the results. Fusion agents could be other chemical fusogens such as PEG 1540 or 4000 or other electrical parameters such as voltage and pulse duration (Davey et al., 2005). However, given that the genotype seems to be one of the most detrimental factors for both fusion and cell proliferation, it seems interesting to concentrate further efforts upon screening first all the available accessions by the electro-fusion technique described here.

Such screening should be completed by cytogenetic studies in order to characterize the DNA content of each accession and to identify the best source material (hypocotyls, leaves, stems, etc.), knowing that protoplast fusion technology is dependent on both DNA content (Davey et al., 2005) and protoplast origin and size (Ochatt et al., 2005; 2007).

Finally, these results should also lean on those obtained with the embryo rescue technique using micro-pod culture (Geerts et al., 2000; 2001; Schryer et al., 2005) to improve the culture media that will give the heterokaryon-derived microcalli the perspective to evolve to hybrid somaclones. Somatic embryogenesis could be performed based on an adaptation of the protocol described by Zambre et al. (2001).

To conclude, this work describes an interesting alternative tool to succeed crosses between PC or PP and PV in order to improve *Phaseolus vulgaris* L. resistance to diseases, in particular and to abiotic or biotic stresses in general. It shows that protoplast fusion technology for *Phaseolus* breeding is a reliable

interesting approach where classical tools, such as embryo rescue, have limited power in the regeneration process of hybrids. This is particularly the case when PC or PP is used as female parent.

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