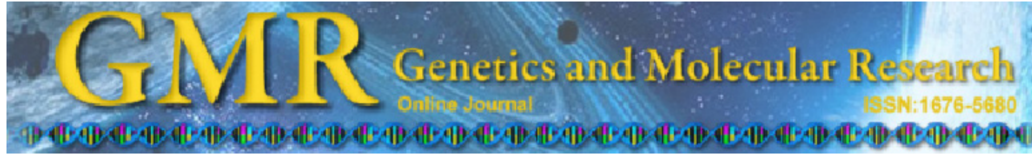

Methodology

A rapid method to increase the number of F_1 plants in pea (*Pisum sativum*) breeding programs

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ABSTRACT. In breeding programs, a large number of F_2 individuals are required to perform the selection process properly, but often few such plants are available. In order to obtain more F_2 seeds, it is necessary to multiply the F_1 plants. We developed a rapid, efficient and reproducible protocol for *in vitro* shoot regeneration and rooting of seeds using 6-benzylaminopurine. To optimize shoot regeneration, basic medium contained Murashige and Skoog (MS) salts with or without B5 Gamborg vitamins and different concentrations of 6-benzylaminopurine (25, 50 and 75 μM) using five genotypes. We found that modified MS (B5 vitamins + 25 μM 6-benzylaminopurine) is suitable for *in vitro* shoot regeneration of pea. Thirty-eight hybrid combinations were transferred onto selected medium to produce shoots that were used for root induction on MS medium supplemented with α-naphthalene-acetic acid. Elongated shoots were developed from all

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hybrid genotypes. This procedure can be used in pea breeding programs and will allow working with a large number of plants even when the F_1 plants produce few seeds.

Key words: Micro-propagation; Growth regulators; Seed culture; Multiple shooting; *In vitro* culture; Hybridization

INTRODUCTION

The garden pea (*Pisum sativum*) is one of the most important pulse crops. Though a number of high-yielding pea varieties have been developed in several countries, there is still a need for materials adapted to different climatic situations. The success of a plant breeding program largely depends on the selection of parents for hybridization and the identification of superior recombinants in the segregating generations. The line x tester analysis method is used for the screening of favorable parents and crosses and the estimation of their general and specific combining abilities (Kempthorne, 1957; Basbag et al., 2007). Sometimes, certain crosses produce fewer F_1 seeds, probably due to differences in flower structure that hinder manual castration. It is possible to artificially increase the number of F_1 individuals by *in vitro* techniques. *In vitro* regeneration studies have been reported for *Pisum*, using different explants, such as cotyledonary nodes (Rajput and Singh, 2010), cotyledons (Pniewsky et al., 2003), immature leaflets (Fujioka et al., 2000), cigotic embryos (Sanchez and Mosquera, 2006), and mature seeds (Zhihui et al., 2009).

We developed and tested *in vitro* system for propagation of F_1 pea genotypes.

MATERIAL AND METHODS

Due to the low numbers of hybrid seeds that were produced, five cultivars of pea (Viper, Canada A, C2001, Marina, and DMR7) were used in different combinations of growing medium and regulators to test which combination produced the best results for micropropagation.

Each experiment consisted of three replicates with 18-20 seeds/replications. Basic medium contained Murashige and Skoog (1962) salts with or without B5 vitamins (Gamborg et al., 1968), 30 g/L sucrose and 6 g/L agar. The pH was adjusted to 5.7 prior to autoclaving (120°C for 20 min). Three concentrations of 6-benzylaminopurine (BA) (25, 50 and 75 μM) were added to the medium before autoclaving. All cultures were incubated in a growth chamber at a temperature of 24°C, a photoperiod of 16 h and 40 $mM \cdot m^{-2} \cdot s^{-1}$ irradiance. Seeds of the pea varieties were surface-sterilized in 70% ethanol for 1 min, subsequently were immersed in 25% commercial sodium hypochlorite solution for 10 min and rinsed twice in sterile water. After four weeks, the number of shoots was recorded and they were transferred onto rooting medium (MS + 0.01 mg/L α-naphthalene-acetic acid). All experiments were repeated twice. After selecting the most appropriate media, seeds of 38 hybrid genotypes were transferred onto the selected medium and were surface-sterilized as described above. The number of shoots was compared at the 5% level using ANOVA of the InfoGen computer program (Balzarini and Di Renzo, 2003). Data were transformed by $\sqrt{x}$ before statistical analysis and grouping of hybrids was performed.

RESULTS AND DISCUSSION

The number of shoots differed significantly between varieties (F = 6.3; P < 0.001) and between basal media (F = 9.4; P < 0.001). The only interaction found was between basal media and BA concentrations. The best combinations were basic medium MS + B5 vitamins + 25 µM BA or basic medium MS + 75 µM BA (Table 1). Due to its lower cost, the medium with the lower concentration of plant regulators would be preferred.

Table 1. Numbers of shoots obtained from different treatments using five pea varieties.

Varieties	MS + 25 µM BAP	MS + 50 µM BAP	MS + 75 µM BAP	MSB5 + 25 µM BAP	MSB5 + 50 µM BAP	MSB5 + 75 µM BAP
C2001	1.33	2.00	9.86	17.00	11.43	3.40
Canada A	2.33	2.00	5.00	8.17	6.86	3.50
Marina	5.00	5.25	19.00	10.67	12.00	12.00
DMR7	12.00	3.50	11.00	20.75	2.50	7.00
Viper	4.33	2.67	21.00	31.50	22.50	5.33

MS = basic medium contained Murashige and Skoog salts, B5 vitamins, 30 g/L sucrose, and 6 g/L agar. BAP = 6-benzylaminopurine.

The number of shoots differed significantly between hybrids (F = 1.9; P < 0.001). A dendrogram showed three main clusters (Figure 1). The first group (12 hybrids) was composed of materials with the highest capacity of shoot formation (25 or more shoots per seed sown), while the second group (16 hybrids) produced between 18 and 22 shoots by explant and the third group (10 hybrids) gave the lowest mean values (less than 18 shoots per explant). In all cases, it was possible to develop elongated shoots from these explants. These shoots were used for root-induction experiments. It was observed that several new shoot buds of different sizes developed at the base of the regenerated shoots at later stages during culture, which increased the final number of regenerated shoots (Figure 2). Two to three weeks was the optimal period of shoot growth for rooting. Root induction at the base of the *in vitro* regenerated shoots was observed when shoots were cultured on MS medium supplemented with 0.01 mg/L α-naphthalene-acetic acid.

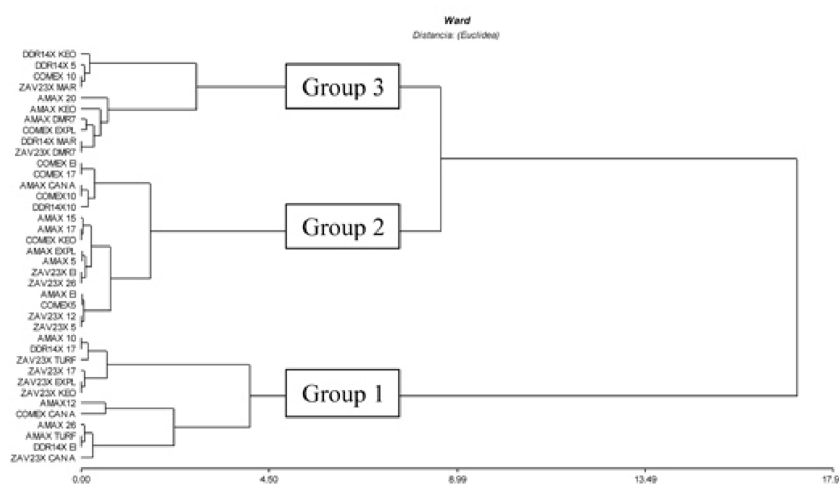

Figure 1. Dendrogram of number of shoots generated from all hybrids.

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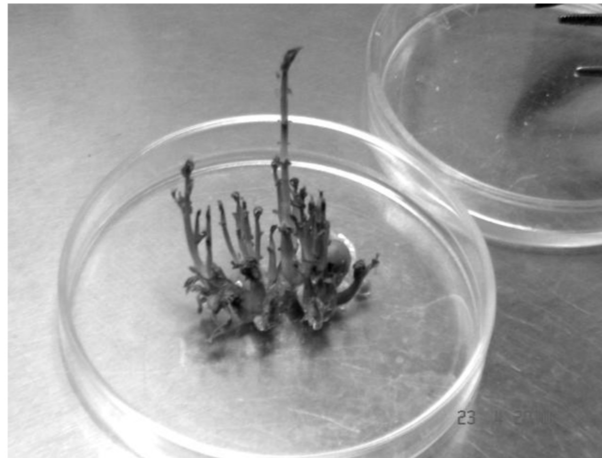

Figure 2. *In vitro* regenerated shoots.

Regenerated plantlets were transplanted to soil in pots. We found that 60% of regenerated pea shoots rooted in this culture medium within six weeks. Generally, the mortality rates were due to soft, weak stems and roots without good differentiation of vessels that are unable to acquire the nutrients from soil when transferred from agar medium. Plantlets were then transplanted to normal greenhouse potting soil and grown to maturity in the greenhouse.

This procedure can be used in pea breeding programs, and will permit working with more plants whenever crosses have low seed production.

REFERENCES

Balzarini M and Di Renzo J (2003). Infogen. Software para Análisis Estadísticos de Marcadores Genéticos. Facultad de Ciencias Agropecuarias. Universidad Nacional de Córdoba, Argentina.

Basbag S, Ekinci R and Gencer O (2007). Combining ability and heterosis for earliness characters in line x tester population of *Gossypium hirsutum* L. *Hereditas* 144: 185-190.

Fujioka T, Fujita M and Iwamoto K (2000). Plant regeneration of Japanese pea cultivars by *in vitro* culture of immature leaflets. *J. Jpn. Soc. Hortic. Sci.* 69: 656-658.

Gamborg OL, Miller RA and Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.

Kempthorne O (1957). An Introduction to Genetic Statistic. Wiley and Sons, New York.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-479.

Pniewsky T, Wachowiak J, Kapusta J and Legocki A (2003). Organogenesis and long-term micropropagations polish pea cultivars. *Acta Soc. Bot. Pol.* 72: 295-302.

Rajput V and Singh NP (2010). Studies on *in vitro* regeneration and direct organogenesis in pea (*Pisum sativum* L.). *Indian J. Plant Physiol.* 15: 246-249.

Sanchez EA and Mosquera T (2006). Establishing a methodology for inducing the regeneration of pea (*Pisum sativum* L.) explants, 'Santa Isabel' variety. *Agron. Colomb.* 24: 17-27.

Zhihui S, Tzitzikas M, Raemakers K, Zhengqiang M, et al. (2009). Effect of TDZ on plant regeneration from mature seeds in pea (*Pisum sativum*). *In Vitro Cell Dev. Biol.* 45: 776-782.

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