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Antigibberellin-induced reduction of internode length favors *in vitro* flowering and seed-set in different pea genotypes

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Abstract

In vitro flowering protocols were developed for a limited number of early flowering pea (*Pisum sativum* L.) cultivars. This work was undertaken to understand the mechanisms regulating *in vitro* flowering and seed-set across a range of pea genotypes. Its final goal is to accelerate the generation cycle for faster breeding novel genotypes. We studied the effects of *in vivo* and *in vitro* applications of the antigibberellin *Flurprimidol* together with radiation of different spectral compositions on intact plants, plants with the meristem removed, or excised shoot tip explants. Based on our results, we present a simple and reliable system to reduce generation time *in vitro* across a range of pea genotypes, including mid and late flowering types. With this protocol, more than five generations per year can be obtained with mid to late flowering genotypes and over six generations per year for early to mid flowering genotypes.

Additional key words: acceleration of development, *Flurprimidol*, gibberellins, spectral composition of radiation.

Introduction

In vitro based modified single-seed-descent (SSD) systems have been proposed as one method to accelerate generation turnover across a number of species (Franklin *et al.* 2000, Ochatt *et al.* 2002, Asawaphan *et al.* 2005, Zhang 2007, Ochatt and Sangwan 2008). The *in vitro* SSD technique involves shortening generation time by culturing immature seeds after forced *in vitro* flowering. Current conventional SSD methodologies enable a maximum of three generations per year to be developed in pea (Ochatt and Sangwan 2010). Pea cultivars are usually released after 8 - 10 generations of self-pollination to achieve an appropriate level of homozygosity (Kasha and Maluszynski 2003). Decreasing the length of the generation cycle will overcome this breeding bottleneck and accelerate genetic improvement. Doubled haploidy is the fastest known route to homozygosity (Chase 1952), however, considerable further research is required before this technology will be available routinely within a pea breeding programme (Croser *et al.* 2006, Ochatt *et al.* 2009). There have been two *in vitro* flowering protocols proposed for pea (Franklin *et al.* 2000, Ochatt *et al.* 2002), however, both protocols were developed for a limited number of early flowering cultivars. To enable the development of a widely applicable protocol, we studied *1*) the effect of *in vivo versus in vitro* application of the antigibberellin *Flurprimidol* to reduce internode length, *2*) the effect of spectral composition of radiation on *in vitro* flowering and seed-set, and *3*) the optimization of culture methodology through comparative *in vitro* cultures of intact plants, plants with the meristem removed, or excised shoot tip explants. Based on our results, we report herewith an improved protocol that enables *in vitro* flowering across a range of pea genotypes, including mid and late flowering types.

The antigibberellin *Flurprimidol* is a chemical used in horticulture to reduce plant height and produce compact plants. It reduces internode elongation through the

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Abbreviations: GA - gibberellic acid; MS - Murashige and Skoog; SSD - single seed descent.
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inhibition of gibberellic acid (GA) biosynthesis (Rademacher 2000). *Flurprimidol* has been extensively used to control plant growth under glasshouse conditions in a number of species (Hamid and Williams 1997, Pobudkiewicz and Treder 2006, Burton *et al.* 2007), including pea (Ochatt *et al.* 2002). However, to our knowledge, *Flurprimidol* has never been used in any *in vitro* flowering protocol by adding it to the medium.

Environmental factors, such as spectral composition of radiation (Reid *et al.* 1996, Cerdan and Chory 2003, Ausín *et al.* 2005, Nelson *et al.* 2010), photoperiod length (Reid *et al.* 1996, Ceunen and Geuns 2013), growth temperature (Ausín *et al.* 2005, Nelson *et al.* 2010), and stresses (Wang *et al.* 2012, Zhou *et al.* 2012) play a key role in the regulation of the transition to flowering in plants. Among these, a mixture of red and far-red radiation is the most effective in causing flowering long day plants (Reid and Murfet 1977, Vince-Prue 1981, Weller *et al.* 1997, Ausín *et al.* 2005, Cummings *et al.* 2007). Runkle and Heins (2001) reported the red/far-red ratio has no effect on *in vivo* flowering time of the long day pea cv. Utrillo. In contrast, Cummings *et al.* (2007) reported the high red/far-red ratio delayed pea flowering *in vivo* and inhibited internode extension in long day genotypes.

To develop a protocol that could be widely adopted, it was necessary to optimize culture factors considered important to *in vitro* flowering and seed initiation and maturation. The factors identified as critical are the gas exchange and air humidity in culture vessels (Lentini *et al.* 1988, Fujioka *et al.* 1999, Asawaphan *et al.* 2005), explant types (Narasimhulu and Reddy 1984, Franklin *et al.* 2000, Ochatt *et al.* 2000, 2002), culture medium composition (Franklin *et al.* 2000, Ochatt *et al.* 2000, 2002, Asawaphan *et al.* 2005), and the addition of plant growth regulators (Narasimhulu and Reddy 1984, Chengalrayan *et al.* 1995, Franklin *et al.* 2000, Ochatt *et al.* 2002). We believe the optimization of these factors would lead to a robust pea *in vitro* flowering protocol.

The aim of this work was to understand the mechanisms operating *in vitro* causing the efficient and reproducible induction of flowering and seed-set across a range of pea genotypes. The final goal is to contribute to the acceleration of generation cycles for faster breeding of novel genotypes.

Materials and methods

A range of genetically diverse *Pisum sativum* L. genotypes with varying flowering times were selected for this research (Table 1).

For *in vivo* experiments, the seeds of cv. Kaspa and line 00P016-1 were sown in a glasshouse at the University of Western Australia (Perth, Australia; lat. 31° 58' 49" S; long. 115° 49' 7" E) in 1 dm^3 pots filled with *UWA Plant Bio Mix* (*Richgro Garden Products*, Perth, Australia). The day/night temperatures were 20/18 °C and natural irradiance. In preliminary experiments, it was observed that cv. Kaspa and line 00P016-1 did not flower under *in vitro* conditions; this was the reason why the *in vivo* approach was taken for these two genotypes.

Flurprimidol [2-methyl-1-pyrimidine-5-yl-1-(4-trifluoro-methoxyphenyl) propane-1-ol] (*Topflor*, *SePRO Corporation*, Carmel, IN, USA), was used to reduce internode elongation. In order to identify the most effective *Flurprimidol* treatment, a 5 % (m/v) solution was applied as a drench at various amounts (0, 25, 50, and 75 cm^3). Applications were repeated three times at 10-day intervals from the three-leaf stage (Ochatt *et al.* 2002).

For *in vitro* experiments, dry seeds were surface-sterilized by 70 % (v/v) ethanol for 5 min, followed by sodium hypochlorite (21 g dm^{-3}) for 10 min. Then, the seeds were rinsed three times with sterile deionised water and imbibed overnight. The coats of the imbibed seeds were removed and 10 embryos with both cotyledons intact were cultured in a vessel containing 50 cm^3 of B5 (Gamborg *et al.* 1968) salts and vitamins modified by the addition of 10 mM NH_4Cl (Ochatt *et al.* 2000). After 7 d, the shoot apical meristems (1 cm length and comprising two internodes) of 50 % of the plants randomly selected were removed using a scalpel. Both the plants with the meristem removed which developed *via* axillary branching and the excised shoot apical meristems were then individually cultured *in vitro* and compared to

Table 1. Pea genotypes used in this study and their main characteristics.

Name	Flowering type	Plant habit	Leaf type	Comment
Frisson	early	semi-dwarf	conventional	French cv. (Ochatt *et al*. 2002)
Excell	early/mid	semi-dwarf	semi-leafless	Australian cv.
Bundi	early/mid	semi-dwarf	semi-leafless	Australian cv.
Victor	mid	semi-dwarf	conventional	French cv. (Ochatt *et al*. 2002)
Dunwa	mid/late	tall	conventional	Australian cv.
Kaspa	late	semi-dwarf	semi-leafless	Australian cv. (industry standard)
00P016-1	very late	tall	conventional	Ethiopian germplasm accession: landrace

cultured intact plants. Three explant types, plants with the meristem removed, excised shoot tips, and intact plants were cultured into either 150 × 25 mm borosilicate glass tubes covered with a polypropylene closure (*PhytoTechnology Laboratories*, Kansas, USA) and containing 20 cm^3 of a Murashige and Skoog (1962; MS) medium, or into 150 × 70 mm polycarbonate containers sealed with a screw cap (*Sarstedt Australia Pty Ltd*, Adelaide, Australia) with a 4 mm diameter hole covered with breathable membrane (*Flora Laboratories*, Melbourne, Australia) with 50 cm^3 of the MS medium. The pH of the medium was adjusted to 5.6 prior to autoclaving at 121 °C for 20 min.

All cultures were incubated at temperature of 24 °C and irradiance of 145 µmol m^{-2} s^{-1} (cool-white-fluorescent tubes *LIFEMAX TL-D* 30W/840, *Philips Lighting*, Bangkok, Thailand) unless stated otherwise. Preliminary experiments included culture of explants at photoperiods of 12, 16, 20, and 24 h. However, there were no significant differences among treatments. Thus, a 20-h photoperiod was chosen for all *in vitro* experiments in this study.

To evaluate the effect of *Flurprimidol* on *in vitro* growth and flowering, a range of amounts (0, 5, 10, 15, 20, 30, and 35 cm^3) of filter-sterilized 5 % (m/v) *Flurprimidol* per dm^3 of the MS medium were added immediately after autoclaving.

To study the effect of spectral composition of radiation on *in vitro* flowering induction, three different radiation sources were compared: *1*) Cool-white fluorescent tubes only (145 µmol m^{-2} s^{-1}); *2*) Cool-white fluorescent tubes combined with red radiation from *F30W/GRO Gro-Lux* tubes (*Sylvania*, Erlangen, Germany) (120 µmol m^{-2} s^{-1}); and *3*) Cool-white fluorescent plus far-red filtered radiation (*Blood Red 789*, *LEE* filters, Andover, England) (130 µmol m^{-2} s^{-1}).

In all experiments, the flowering time and node number of the first flower were recorded. Also, at day 40 of culture, plant length and number of nodes were measured and then divided to get the average internode length. In addition, the efficiency of recovery of immature seeds and embryos was studied. This included the selection of the embryo developmental stage (number of days after flowering) for best *in vitro* germination and plant development. The immature pods at 16, 18, 20, and 22 d after flowering were opened aseptically and the embryos removed and directly transferred onto new glass tubes containing 20 cm^3 of the hormone-free MS medium.

The experimental design was completely randomized and all treatments were repeated at least three times with a minimum of 10 replicates per treatment per genotype. Statistical analysis was performed by analysis of variance (*Microsoft Office Excel 2007* software).

Results

The pea plants *in vivo* were smaller after the addition of *Flurprimidol* due to reduced internode length (Fig. 1*A*,*B*). For example, for the line 00P016-1, we observed an average reduction in plant height from 77 ± 7.0 cm in the control treatment to 13 ± 2.8 cm for the highest *Flurprimidol* concentration applied. *Flurprimidol* also reduced internode length *in vitro* (Table 2) but it had no effect on a node number, flowering time, and seed-set under either *in vivo* or *in vitro* conditions.

For both *in vivo* and *in vitro* experiments, an important seasonal effect was detected. In the glasshouse under natural irradiance, internode length was reduced by *Flurprimidol* more in spring than in autumn. For any given concentration used, *Flurprimidol* was less effective in reducing plant size during the autumn-winter period (shorter days and lower irradiance) than in the spring-summer period (longer days and higher irradiance) (Fig. 1*A*). Similarly, in controlled environment rooms under low irradiance (110 - 180 µmol m^{-2} s^{-1}) *Flurprimidol* was less effective in reducing plant size *in vitro*, therefore, a higher concentration was required than under higher irradiance (550 - 900 µmol m^{-2} s^{-1}) to produce similar effects.

The effectiveness of *Flurprimidol* on reducing plant height varied across the genotypes. *Flurprimidol* had a greater effect on the tall genotypes compared to the semi-dwarf genotypes. For example, the *Flurprimidol* solution reduced plant height of the tall landrace 00P016-1 by 73.6 % compared to the control, whereas the same *Flurprimidol* treatment reduced plant height only by 37.9 % in the semi-dwarf cv. Kaspa.

Table 2. Effect of 5 % (m/v) *Flurprimidol* applied at different amounts into a medium on the internode length of pea cv. Kaspa grown *in vitro* from different explants for 40 d. Means ± SE, $n = 30$; different letters indicate means significantly different at $P \leq 0.05$.

Explants	*Flurprimidol* [cm^3 dm^{-3}]	Internode length [mm]
Intact plants	0	13.8 ± 0.17a
	15	7.1 ± 0.21b
	30	6.8 ± 0.33b
Meristem removed	0	13.6 ± 0.18a
	15	9.2 ± 0.28b
	20	9.1 ± 0.25b
	30	5.9 ± 0.13c
	35	5.9 ± 0.40c
Shoot tip explants	0	5.4 ± 0.31a
	5	4.9 ± 0.15a
	10	3.6 ± 0.41b
	15	3.4 ± 0.23b
	20	3.5 ± 0.10b
	30	3.5 ± 0.15b

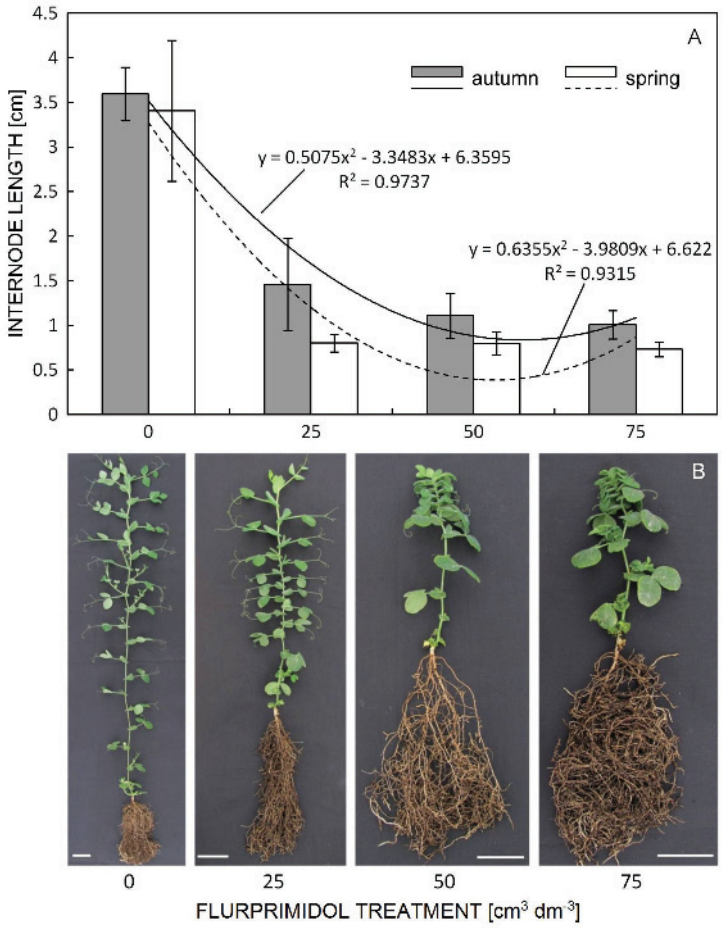

Fig. 1. Effect of *Flurprimidol* treatments [5 % (m/v) solution was applied as a drench at various amounts (0, 25, 50, and 75 cm^3)] on pea landrace 00P016-1 grown in a glasshouse in dependence on season. *A* - Internode length in autumn or spring (means ± SD; *n* = 30). *B* - Plant size and morphology (*bars* = 5 cm).

Flurprimidol reduced *in vitro* the internode length in dependence on the used explant type: intact plants were less affected than the plants with the meristem removed or excised shoot tip explants (Table 2). Plants with the meristem removed developed through the proliferation of a cotyledonary node axillary meristem. No significant difference was observed on *in vitro* time to flowering when comparing the intact plants and the plants with the meristem removed. Thus, for the plants of cv. Excell grown in the tubes, the average flowering time was 32 ± 2.7 d for the intact plants and 32 ± 5.9 d for the plants with the meristem removed. In contrast, the excised shoot tip explants cultured *in vitro* always took longer to flowering, *e.g.*, the average flowering time of the excised shoot tip explants of cv. Excell grown in the tubes was 44 ± 5.5 d. It was observed that only the excised shoot tip explants that produced roots were able to form flowers.

No significant effect of spectral composition of radiation was observed on days to flowering, percentage of flowering plants, and percentage of plants with pods. White fluorescent tubes were thus used in all other experiments. In general, there was a higher percentage of seed-set in the plants grown in the tubes compared to the plants grown in the containers (Table 3), despite more flowers being produced in the containers (Table 4). The strongest difference between the treatments was observed for conventional leafed cvs. Frisson and Victor. However, a lower difference between the treatments was observed

Table 3. Percentage of plantlets that set seeds. Pea plantlets were grown *in vitro* from different explants either in tubes (T) or in containers (C). Means ± SE, *n* = 30.

Genotype	Vessel	Meristem removed	Shoot tip explants	Intact plants
Victor	T	96.6 ± 1.04	40.0 ± 1.06	93.3 ± 1.04
	C	66.7 ± 1.18	0.0 ± 0	70.0 ± 1.40
Frisson	T	90.6 ± 1.66	54.2 ± 0.75	90.9 ± 1.15
	C	60.0 ± 1.29	66.7 ± 1.22	57.1 ± 1.64
Excell	T	62.5 ± 1.91	75.0 ± 1.68	55.6 ± 0.98
	C	57.1 ± 1.00	30.0 ± 1.09	6.1 ± 1.31
Bundi	T	72.0 ± 1.37	44.5 ± 1.69	20.0 ± 1.55
	C	56.5 ± 1.86	35.5 ± 1.73	25.0 ± 1.00

Table 4. Number of flowers per plantlet of cvs. Frisson and Excell produced *in vitro* from different explants either in tubes (T) or in containers (C). A volume of 30 cm^3 (intact plants and plants with the meristem removed) or 10 cm^3 (shoot tip explants) of 5 % (m/v) *Flurprimidol* was applied per 1 dm^3 of a medium. Means ± SE, n = 30; different letters indicate means significantly different at $P \leq 0.05$.

Explant	Frisson		Excell	
	T	C	T	C
Meristem removed	1.60 ± 0.11a	2.70 ± 0.14c	1.25 ± 0.07a	1.73 ± 0.19bc
Shoot tip explants	1.07 ± 0.05b	1.60 ± 0.16a	1.07 ± 0.05a	1.30 ± 0.13ab
Intact plants	1.46 ± 0.11a	2.64 ± 0.22c	1.16 ± 0.07a	1.93 ± 0.16c

for semi-leafless cvs. Excell and Bundi. Interestingly, for intact plants of cvs. Excell and Bundi, the containers favoured marginally better seed-set than the tubes. The type of the culture vessel used had no effect on plant architecture irrespectively of the genotype studied.

In order to shorten the length of the reproductive cycle, immature embryos were removed and cultured *in vitro*. Best germination rates were obtained by culturing immature seeds between 18 - 20 d after flowering. The length of the generation cycle *in vitro* was about 50 d for most of the genotypes studied (Victor: 48 ± 1.8 d; Bundi: 49 ± 3.6 d; Excell: 50 ± 5.9 d; and Frisson: 54 ± 3.4 d). On the other hand, for the very late flowering landrace 00P016-1, the average generation length *in vitro* was over 90 d with a very low flowering and seed setting rate (under 10 %).

Table 5. Number of generations per year and flowering time in days (in brackets) as affected by pea genotype and environmental conditions. Means from three repetitions with at least 10 individuals. Field: Department of Agriculture and Food of Western Australia, Australia.

Environment	Dunwa	Frisson	Bundi	Excell
Field	2.0 (91)	2.0 (75)	2.0 (85)	2.0 (85)
Glasshouse	3.6 (62)	4.1 (64)	3.7 (56)	3.8 (54)
In vitro	5.2 (50)	6.6 (36)	7.0 (31)	7.1 (31)

Significant differences among the studied genotypes were observed in flowering time and in the estimated average generation cycle length when comparing the plants grown under the different growth environments (Table 5). With the proposed *in vitro* flowering protocol, up to seven generations per year can be obtained for the early/mid flowering cvs. Bundi and Excell; and over five generations for the mid/late flowering cv. Dunwa.

Discussion

An optimized *in vitro* flowering protocol is presented here using *Flurprimidol* to control *in vitro* plant size. *In vitro* flowering and seed-set was achieved in diverse pea genotypes with an average generation cycle length of approximately 50 d for the early to mid flowering cultivars. We confirmed our hypothesis that the antigibberellin *Flurprimidol* stimulates *in vitro* flowering by reducing internode length. However, the addition of red or far-red radiation did not reduce the flowering time *in vitro* in the genotypes studied.

In order to shorten the generation cycle of pea, it is essential to control plant growth to obtain plants with reduced vegetative development (Ochatt *et al.* 2002). In horticulture, *Flurprimidol* has been used *in vivo* to produce compact plants. This chemical acts by blocking cytochrome P_{450}-dependent monooxygenases, catalyzing the oxidation of ent-kaurene into ent-kaurenoic acid, thereby inhibiting GA biosynthesis (Rademacher 2000). In our experiments, *Flurprimidol* reduced pea plant size *in vivo* and *in vitro*, which is particularly important for tall genotypes.

Internode length in pea is determined at least by five major loci: *Le, La, Cry, Na, Lm* (Reid *et al.* 1983). GA_1 is the native GA controlling internode elongation factor in pea (Ross *et al.* 1989). Slender phenotypes (*la, cry*) have long and thin internodes and are not dependent on endogenous GAs. Dwarf plants (*La* and/or *Cry*) acquire a similar phenotype to slender types when treated with high concentrations of GA_3 (Potts *et al.* 1985). A major gene (*Le/le*) for internode length is mainly responsible for tall climbing (*Le*/-) *versus* dwarf bush (*le/le*) habit (Potts *et al.* 1982). Application of GA_1 can mask the *Le/le* gene difference. *Le* plants respond equally to GA_{20} and GA_1, while *le* plants respond only weakly to GA_{20}, the major biologically active gibberellin found in dwarf peas. These results suggest that the *Le* gene controls the production of a 3β-hydroxylase capable of converting GA_{20} to GA_1 (Ingram *et al.* 1983).

Gibberellins are a large group of compounds which share a common gibbane ring structure and which all have at least some physiological activity (Cleland 1999). They include both precursors and catabolites and thus, even if GA_1 is the main active GA in stem elongation (Ingram *et al.* 1986), other GAs are as active or more active in processes including, among others, tendril and pod growth in pea (Smith *et al.* 1992). Thus, whereas the

biosynthesis of GA_{20} takes place in unfolded leaves and tendrils, its conversion to GA_1 occurs in the upper stem (Smith 1992). The paramount role of GAs on leaf expansion has been proven in cereals (Nelissen *et al.* 2012) and is also known in pea (Hedden 1999). In this context, the differential responses observed among the different tissues tested and also among the conventional (Frisson and Victor) and semi-leafless (Bundi and Excell) cultivars might be attributed to a differential endogenous content of active GAs within the tissues in conjunction with the antigibberellin effect of the exogenously applied *Flurprimidol*.

For long-day plants, a red to far-red ratio close to the natural level is the most effective for flower induction (Vince-Prue 1981). Most growth chamber sources of radiation have high red/far-red ratios (greater than 2) which can delay flowering and inhibit internode extension (Whitman *et al.* 1998, Runkle and Heins 2001, Cummings *et al.* 2007). In our experiments, the addition of red or far-red radiation did not reduce the flowering time *in vitro* in the early, mid or late flowering pea genotypes. The addition of far-red filtered radiation probably did not correct the red/far-red ratio sufficiently to mimic natural irradiance, as it was discussed by Runkle and Heins (2001) and Cummings *et al*. (2007). Also, the irradiance may not have been high enough to induce fast *in vitro* flowering in the genotypes studied (Fujioka *et al.* 1999). It was then concluded that white fluorescent tubes were the best radiation sources for the *in vitro* flowering protocol presented here.

Growth rates and many of the physiological characteristics of plants developed *in vitro* are influenced by the physical and chemical environment of culture vessels (Walker *et al.* 1988, Jackson *et al.* 1991, Kozai *et al.* 1992, Majada *et al.* 1997). The type of culture vessel and its closure (use of gas-permeable membranes or lids) influences the *in vitro* environment by modifying the gas composition. When comparing different culture vessels, we observed a higher percentage of the seed-setting plants when culturing in the tubes covered with polypropylene caps compared to the containers sealed with screw caps covered by a breathable membrane. This is likely to be associated with better air exchange (reduced concentration of ethylene and higher CO_2 concentration) in glass tubes compared to polycarbonate containers, as reported by Lentini *et al*. (1988) and Jackson *et al.* (1991). Interestingly, we observed that the plants grown in the tubes produced a lower number of flowers compared to the plants grown in the containers, and that this was true irrespectively of their flowering or leaf type, as a similar trend was observed in the leafy type cv. Frisson compared to the early/mid flowering semileafless type cv. Excell. This might be related to the smaller amount of the culture medium used in the tubes compared to the containers, which might affect the supply of nutrients, thus affecting the production of flowers, as observed previously with other species (Figueira and Janick 1994, George *et al.* 2009). Therefore, the tubes were considered the best culture vessel in the *in vitro* flowering protocol proposed in this work, as the reduced number of flowers per plant produced was also associated with lesser early pod abortion, maybe due to reduced competition between flowers.

Efficient breeding methods are needed to advance hybrid populations and to facilitate selection of lines with desirable combinations of characters (Haddad and Muehlbauer 1981). The SSD method consists of taking a single seed from each F_2 plant and advancing each seed to the next generation until a desired level of homozygosity is achieved (F_6 to F_8), thus saving space and time (Goulden 1939). However, the extent of plant loss from generation to generation affects the genetic makeup of the SSD populations (Martin *et al.* 1978). We have calculated that with an attrition rate per generation of 10 % of the plant population after four generations of SSD (from F_2 to F_6) we would end up with only 34 % of the initial population. This indicates the importance for the breeder of the robustness and reliability of any tissue culture system used. In the present study, removing the meristem and culturing it separately permits the production of a second cloned plant, which provides a back-up plant in case of loss. This is particularly important when working with rare and valuable genotypes in a population such as the production of recombinant inbred lines (Soller and Beckmann 1990).

The identification of a system to accelerate generation turnover by shortening each cycle is crucial in breeding programmes. In this publication we present a simple and reliable system to reduce generation time *in vitro* across a range of pea genotypes. In this protocol, *Flurprimidol* was used to control plant size *in vitro*, and the plants with the meristem removed, and the shoot tip explants were cultured in the glass tubes. More than five generations per year could be obtained with mid to late flowering genotypes using this protocol, and over six generations per year for early to mid flowering genotypes. However, some late/very late flowering genotypes like cv. Kaspa and the landrace accession 00P016-1 remain recalcitrant to this technology.

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