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PLANT DEVELOPMENT/REGENERATION

Plant growth regulators improve *in vitro* flowering and rapid generation advancement in lentil and faba bean

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Abstract Rapid generation technology (RGT) involves applying plant growth regulators to accelerate *in vitro* flowering and the use of immature seed to shorten the time required to produce the next generation of plants. The effect of different concentrations of flurprimidol and combinations of one cytokinin and two auxins on *in vitro* flowering was evaluated using two lentil (*Lens culinaris* Medik.) and two faba bean (*Vicia faba* L.) cultivars. Adding flurprimidol to the medium reduced the internode length of plants, and plant height was decreased to approximately 10 cm in both species. The optimal concentration of flurprimidol depended on the species and the light intensity. The combination of 0.3 μM flurprimidol, 5.7 μM indole-3-acetic acid, and 2.3 μM zeatin resulted in 100% of faba bean plants flowering and 90% setting seed. The combination of 0.9 μM flurprimidol, 0.05 μM 4-chloroindole-3-acetic acid, and with a perlite growth substrate resulted in 90% of lentil plants flowering and over 80% with seed set. However, faba bean showed better response with agar as the substrate. Under optimized conditions, a single generation cycle was achieved in 54 d for faba bean and 45 d for lentil. RGT could produce seven and eight generations per year for faba bean and lentil, respectively. For the single seed descent breeding method for self-pollinated plants, a seed of each plant in each generation is advanced three times per year until near-homozygosity, thus requiring more than 2 yr. The RGT method produces about double the number of generations per year and therefore has potential for significant acceleration of pulse crop breeding programs.

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Introduction

Rapid generation technology (RGT) is based on the principle of growing miniature plants in an artificial medium under controlled conditions, and allowing them to produce a few flowers which develop seeds that are harvested prior to normal seed maturity, thus reducing the duration of the breeding cycle. The length of the breeding cycle from seed to seed is often a limiting factor in plant breeding, in the development of recombinant inbred lines (RILs) required for genetic analysis, and in the exploitation of molecular marker technology (Ochatt *et al.* 2002; Ochatt and Sangwan 2008).

Exogenous application of growth regulators improved plant growth and seed setting in mung bean (Ali *et al.* 2008). Combinations of auxin and cytokinin accelerated flowering in white lupin (El-Saeid *et al.* 2011) and lentil (Khalil *et al.* 2006). Although Khalil *et al.* (2006) used kinetin for control of flower abscission of lentil, they concluded that zeatin was the more active cytokinin. *cis*-Zeatin activity has been shown in other plants (Bassil *et al.* 1993; Yonekura-Sakakibara *et al.* 2004). Flurprimidol decreased the length of internodes and vegetative growth in field pea and grass pea (Ochatt *et al.* 2002), thus allowing growth of plants in small *in vitro* containers. However, addition of flurprimidol to the medium had no effect on *in vitro* flowering time of field pea (Ochatt *et al.* 2002; Ribalta *et al.* 2014).

The objectives of this study were to optimize *in vitro* growing conditions for lentil and faba bean *via* adjusting the application of growth regulators (indole acetic acid (IAA), 4-chloroindole-3-acetic acid (4-CL-IAA), zeatin, and flurprimidol), and selecting an appropriate growth medium

(Murashige and Skoog (MS; Murashige and Skoog 1962), chickpea medium (ChM1; Croser *et al.* 2011), and a commercial hydroponic solution (HS)) and substrate (agar or perlite), to reduce the time to flowering and enhance the flowering and seed setting rates.

Material and Methods

Plant material and growth conditions. The lentil cultivars 'CDC Maxim' and 'CDC Greenland,' and the faba bean cultivars 'FB 9-4' and 'CDC Snowdrop' were used (Table 1). All four cultivars were developed by the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada.

Mature lentil and faba bean seeds were washed using Tween 80 (0.1 mL/L) for 4 min before sterilization. Seeds were surface-sterilized by immersion in $Ca(ClO)_2$ at 15 g/L for 20 min then rinsed two to three times in sterile water and soaked for 8 h. Then seeds were completely drained and kept in an incubator (24°C) in darkness overnight. The following day, seeds were washed with sterile water another two to three times, drained, and returned to the incubator. After emergence of the root and shoot, which took place within 2–3 d for lentil and 6–7 d for faba bean, seedlings were transferred to an incubator with 22/18°C day/night temperature and 178 µmol m^{-2} s^{-1} light intensity with 18-h light/6-h darkness. The germinating seeds were then cultured for 3 d on medium solidified with agar (A7002, Sigma-Aldrich Corp., St. Louis, MO), consisting of modified MS medium with half-strength ammonium nitrate and MS microelements, plus 30 g/L sucrose (pH 6.5). The sucrose concentration was increased to 40 g/L for faba bean in modified ChM1 medium (Table 2). The plants were then cultured in double Magenta™ boxes with 19 cm height. The temperature was controlled at 20/18°C day/night, with 20-h light and 4-h darkness.

A commercial fertilizer (Holland's Secret of PlantLife Products™, Kelowna, Canada) used in combination with either agar or perlite (horticulture medium grade, Supreme, Portland, OR) as a substrate was compared with MS and ChM1 media. For seedlings in perlite, the HS 'vegetative formula' was used up to flower initiation, followed by the 'ripening formula,' according to manufacturer's instructions (Table 2). The vegetative formula consisted of 2.5 mL/L Grow, 1.25 mL/L Micro, and 0.75 mL/L Bloom while the ripening formula consisted of 0.25 mL/L Grow, 1.75 mL/L Micro, and 5 mL/L Bloom (formula was based on HS instructions).

Light quality. For the lentil experiments, T5 (841, Philips, Amsterdam, The Netherlands) cool white fluorescent tubes with PAR at 500 µmol s^{-1} m^{-2} and red (655–665 nm) to far-red (725–735 nm) ratio of 1.9 were used. The red to far-red ratio was assessed using a radio spectrometer (Apogee, PS-200, Logan, UT). For the faba bean experiments, the light intensity of T5 cool white fluorescent tubes was adjusted to PAR 600 µmol m^{-2} s^{-1} with a red to far-red ratio of 5.3. In both lentil and faba bean experiments, the effect of flurprimidol under similar light qualities and intensity was evaluated.

Growth regulators. Flurprimidol (Topflor™ containing 0.38% [*w/v*] [α-(1-methylethyl)-alpha-(4-(trifluoromethoxy) phenyl)-5-pyrimidinemethanol]; SePRO Corporation, Carmel, IN) as well as various auxins (Sigma) and *cis/trans* zeatin mix (approx. 80% *trans*; balance primarily *cis*; Sigma) were sterilized by filtration through a 0.2-µm Dyngard membrane (Nalgene, Rochester, NY) and added to the sterile medium. *Cis/trans* zeatin (mix) was chosen because *cis*-zeatin has a growth-promoting role in pea and chickpea, and during embryogenesis and seed development (Bassil *et al.* 1993; Emery *et al.* 1998; Quesnelle and Emery 2007). Recently, Slater *et al.* (2013) also found *cis*-zeatin riboside present in lentil and faba bean seeds 4–12 d after anthesis.

For lentil, different concentrations of flurprimidol (0.3, 0.6, 0.9, or 1.5 µM), IAA (5.7 µM), 4-Cl-IAA (0.05, 0.48, or 4.8 µM), and *cis/trans* zeatin (0.5, 0.9, 4.5, or 9.1 µM) were used. In order to select the best ratio of auxin and cytokinin for faba bean *in vitro* flowering, flurprimidol (0.3, 0.4, or 0.5 µM), IAA (5.7 µM), 4-Cl-IAA (0.05 or 0.48 µM), and *cis/trans* zeatin (0, 0.9, 2.3, or 4.5 µM) were compared.

In addition, the effect of medium composition (½MS, ChM1, or HS; Table 2) and substrate (agar or perlite) with

Table 1. Description of the four cultivars used in the assessment of *in vitro* flowering in lentil and faba bean

Crop	Cultivar	Flower color	Cotyledon color	Seed coat color	Seed weight (mg)	Days to maturity in the field[z]	Generation /year in the growth chamber[y]
Lentil	CDC Maxim	White	Red	Gray	40	102	3
Lentil	CDC Greenland	White	Yellow	Green	64	107	3
Faba bean	CDC FB 9-4	White with black spots	Yellow	Tan	680	104	3
Faba bean	CDC Snowdrop	White	Yellow	White	335	104	3

[z] From germination to harvest

[y] Without applying Rapid Generation Technology protocol

Table 2. Media composition used in rapid generation technology for lentil and faba bean

Macro elements	Modified MS for lentil (mg/L)	Modified ChM1[z] for faba bean (mg/L)	Modified B5[y] for embryo rescue (mg/L)	Commercial hydroponic fertilizer (HS)[x]	
NH_4NO_3	825	0	0	Grow	
KNO_3	1,900	2,500	2,500	Vegetative	Ripen
$CaCl_2{\cdot}2H_2O$	440	600	150	2.5 mL/L	0.25 mL/L
$MgSO_4{\cdot}7H_2O$	370	370	250		
KH_2PO_4	170	170	134		
$NaH_2PO_2{\cdot}H_2O$	0	0	150		
NH_4Cl	0	0	535		
NaFeEDTA	27.85	27.85	27.85		
Microelements				Micro	
KI	0.83	0.83	0.75	Vegetative	Ripen
H_3BO_3	6.20	6.20	3.00	1.25 mL/L	1.75 mL/L
$MnSO_4{\cdot}H_2O$	16.91	16.91	10.00		
$ZnSO_4{\cdot}7H_2O$	8.60	10.00	2.00		
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25		
$CuSO_4{\cdot}5H_2O$	0.025	0.05	0.025		
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025		
Vitamins				Bloom	
Myo-Inositol	100.00	100.00	100.00	Vegetative	Ripen
Pyridoxine HCl	1.00	0.50	1.00	0.75 mL/L	5 mL/L
Thiamine HCl	10.00	0.50	1.00		
Nicotinic acid	1.00	5.00	10.00		
Sucrose	30 g/L	40 g/L	30 g/L	0	
pH	6.5	6.5	6.5	6.5	
Agar	6 g/L	6 g/L	6 g/L	6 g/L agar or 100 mL/ Magenta box perlite	
PGR					
IAA	5.7 μM	5.7 μM	1 μM	5.7 μM	
4-Cl-IAA	0.05, 0.48, 4.77 μM	0.05, 0.48 μM	0.95 μM	0.05, 0.48 μM	
cis/trans Zeatin (mix)	0.5, 0.9, 4.5, 9.1 μM	0, 0.9, 2.3, 4.5 μM	0.9 μM	2.3 μM for faba bean	
Flurprimidol	0.3, 0.6, 0.9, 1.5 μM	0.3, 0.4, 0.5 μM	NA	0.3 μM for faba bean	

[z] This medium was modified based on Croser *et al.* 2011

[y] This medium was modified based on Gamborg *et al.* 1968

[x] Holland's Secret fertilizer™ (Plant Life Products, Kelowna, Canada)

the optimal plant growth regulator (PGR) mixture was evaluated. The combination of ½MS (for lentil) and ChM1 (for faba bean) and agar was defined as tissue culture method. Applying perlite with HS was defined as the hydroponic method. Lastly, an intermediate method was defined as the combination of HS as medium and agar as substrate. Once seeds approached physiological maturity (12–14 d after flowering for lentil, 17–18 d after flowering for faba bean), they were separated from the pods and transferred to modified B5 medium (Gamborg *et al.* 1968) in order to facilitate germination in sterile conditions (Table 2).

Measurements. The vegetative characteristics (plant height, number of nodes, and number of branches), reproductive characteristics (flowering time and rate, and percentage of plants that flowered), and the seed set rate (percentage of plants that produced at least one seed) were recorded. The generation cycle time was calculated from the day when seeds were sterilized to the day of embryo removal from the pod in order to allow comparison with field grown material.

Statistical analysis. A completely randomized design was used. Each experiment was repeated two times with a minimum of 10 replications per treatment per cultivar. The two experimental runs were combined after confirming the homogeneity of variance using Levene's test (Levene 1960). For homogeneity, the values of 0 and 100 were converted into ¼n and 100–¼n (n=number of replication in each treatment; McDonald 2009). The final means presented in the results are the combination of two cultivars and two experimental runs. All data were subjected to analysis of variance (ANOVA) and orthogonal contrasts using PROG GLM in SAS statistical program (SAS Institute Inc. 2013, Cary, NC; Sinclair 2011).

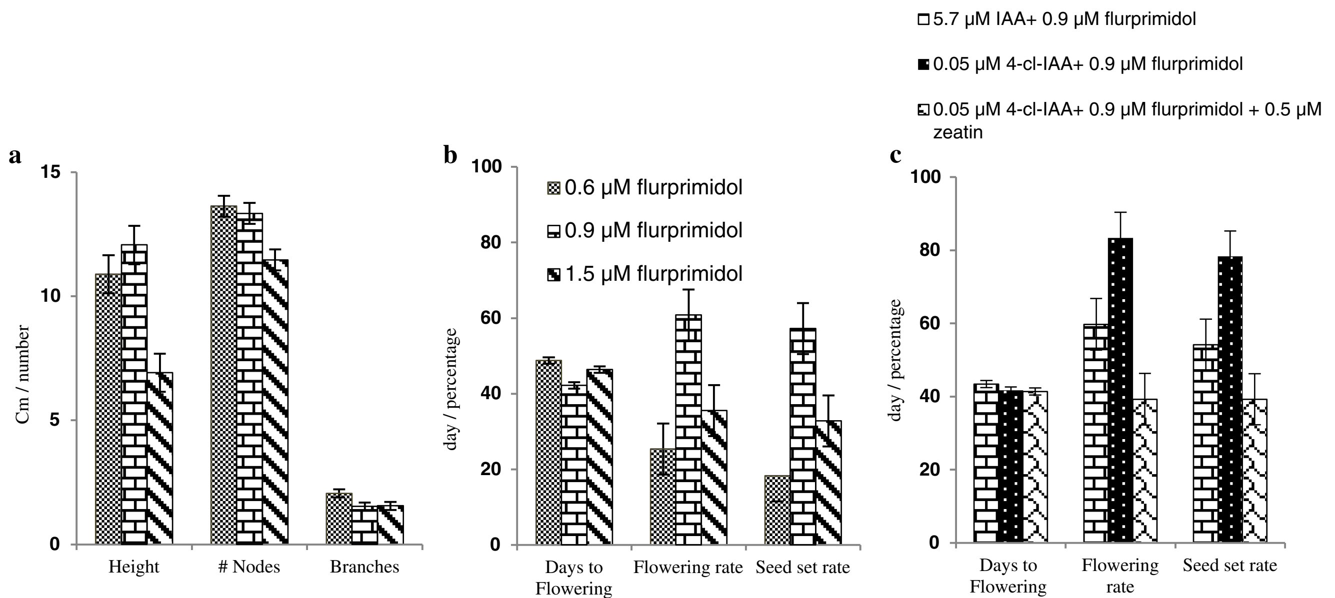

Figure 1. Effect of plant growth regulators (flurprimidol, IAA, 4-Cl-IAA, and zeatin) on vegetative (*A*) and reproductive (*B*, *C*) traits of lentil (average of 'CDC Maxim' and 'CDC Greenland') under PAR=500 μmol m^{-2} s^{-1} in ½MS medium with agar. *Error bars* indicate standard error ($P<0.05$).

Results

Effect of flurprimidol on lentil growth in vitro. The size of plants was adjusted for growth in double Magenta boxes using 5% Topflor™ (with flurprimidol as the active ingredient). Adding flurprimidol to the medium reduced the internode length of plants, and plant height was decreased. The effect of flurprimidol on plant height was shown to be inversely influenced by light intensity (Ribalta *et al.* 2014). Therefore, the flurprimidol concentration was optimized based on the light intensity. Using a light intensity of 500 μmol m^{-2} s^{-1}, the chosen concentration of flurprimidol was 0.6 and 0.9 μM. These two concentrations adjusted the plant height to 10 and 12 cm, respectively, so that lentil could be grown in double Magenta boxes. The number of nodes was higher at these concentrations compared to 1.5 μM flurprimidol (Fig. 1*A*). Flowering and seed set rate increased, while time to flowering decreased significantly by adding 0.9 μM of flurprimidol to the medium compared to 0.6 and 1.5 μM (Fig. 1*B*). Thus, adding 0.9 μM flurprimidol to the medium was more suitable for improving flowering under these light conditions.

Using 0.9 μM flurprimidol, in both lentil cultivars, flowering and seed set rates were significantly increased by 44.4 and 45.8%, respectively, compared to 0.6 or 1.5 μM flurprimidol. Moreover, flowering was accelerated by an average of 5.8 d compared to 0.6 or 1.5 μM flurprimidol (Table 3, *C3*).

Effect of auxin on lentil growth in vitro. Initially, the effect of three concentrations of 4-Cl-IAA (0.05, 0.48, or 4.77 μM) or IAA (5.7 μM) using modified MS medium containing 0.9 μM flurprimidol was determined for rates of flower induction and seed set. The results showed that 0.05 μM 4-Cl-IAA was the most effective treatment in terms of increasing the average

Table 3. Effect of plant growth regulators on increase or decrease of lentil *in vitro* flowering characteristics using orthogonal contrasts

Contrast	Plant growth regulator group	Days to flowering	Flowering rate (%)	Seed setting rate (%)
C1	0.9 *vs.* 0.6 μM flurprimidol	-7.9^{***}	49.9^{***}	52.7^{***}
C2	0.6 *vs.* 1.5 μM flurprimidol	-3.7^{**}	38.8^{***}	38.8^{***}
C3	0.9 *vs.* 0.6 and 1.5 μM flurprimidol	-5.8^{***}	44.4^{***}	45.8^{***}
C4	0.05 μM 4-Cl-IAA *vs.* 5.7 μM IAA	-3.5^{*}	43.2^{***}	46.2^{***}
C5	5.7 μM IAA + 0.5 μM zeatin *vs.* 5.7 μM IAA	-1.3^{ns}	1.5^{ns}	7.3^{ns}
C6	0.05 μM 4-Cl-IAA *vs.* 0.05 μM 4-Cl-IAA + 0.5 μM zeatin	-2.2^{ns}	41.6^{***}	38.8^{***}
C7	0.05 μM 4-Cl-IAA *vs.* other PGRs	-2.8^{*}	42.4^{***}	42.5^{***}

Data presented as delta value of means of cultivars CDC Maxim and CDC Greenland. Contrast C4–C7 included medium with 0.9 μM flurprimidol

ns orthogonal contrast was not significant

$^{*}P$=0.05; $^{**}P$=0.01; $^{***}P$=0.001 (orthogonal contrast was significant)

Table 4. Effect of culture method on increase or decrease of lentil *in vitro* flowering characteristics using orthogonal contrasts

Contrast	Culture method group	Days to flowering	Flowering rate (%)	Seed setting rate (%)
C1	CDC Greenland *vs.* CDC Maxim	0.5^{ns}	6.8^{*}	11.8^{**}
C2	HS *vs.* ½MS	-3.5^{***}	21.2^{***}	11.2^{**}
C3	Perlite *vs.* agar	-4.7^{***}	18.4^{***}	19.1^{***}
C4	0.05 μM 4-Cl-IAA *vs.* 5.7 μM IAA	-7.2^{***}	21.1^{***}	51.1^{***}
C5	Perlite + 5.7 μM IAA *vs.* agar + 5.7 μM IAA	-3.5^{***}	27.3^{***}	11.9^{*}
C6	Perlite + 0.05 μM 4-Cl-IAA *vs.* agar + 0.05 μM 4-Cl-IAA	-5.9^{***}	9.5^{*}	26.4^{**}
C7	HS + perlite + 0.05 μM 4-Cl-IAA *vs.* HS + perlite + 5.7 μM IAA	-8.8^{***}	9.2^{ns}	60.8^{***}
C8	HS + perlite + 0.05 μM 4-Cl-IAA *vs.* other treatments	-9.0^{***}	20.3^{*}	51.8^{***}
C9	Intermediate method *vs.* tissue culture method	-1.5^{**}	16.1^{***}	2.2^{ns}
C10	Hydroponics method *vs.* intermediate method	-3.9^{***}	10.4^{*}	18^{**}
C11	Hydroponics method *vs.* tissue culture method	-5.4^{***}	26.4^{***}	20.2^{***}

Data presented as delta values of means of cultivars CDC Maxim and CDC Greenland. All contrasts included medium with 0.9 μM flurprimidol

ns orthogonal contrast was not significant

$^{*}P=0.05$; $^{**}P=0.01$; $^{***}P=0.001$ (orthogonal contrast was significant)

flowering rate for the two lentil cultivars (Fig. 1*C*). The combination of 0.05 μM 4-Cl-IAA and 0.9 μM flurprimidol led to a significant increase in the average flowering and seed setting rates by 43.1 and 46.1%, respectively, compared to 5.7 μM IAA, while the average time to flowering decreased by 3.5 d in this treatment (Table 3, *C4*).

The three levels of zeatin tested (0.5, 0.9, 2.3, or 9.1 μM) with 5.7 μM IAA did not enhance the lentil flowering or seed set rates. Likewise, adding 0.5 μM zeatin in combination with 0.05 μM 4-Cl-IAA did not affect flowering time and decreased flowering and seed setting rate compared to 0.05 μM 4-Cl-IAA treatment without zeatin (Fig. 1*C*; Table 3, *C6*).

The effect of auxin, zeatin, substrates (perlite or agar), and nutrient solutions (½MS or HS) on lentil flowering characteristics were compared for three different culture methods: HS using perlite, an intermediate method of agar + HS, and tissue culture of ½MS + agar (Table 4). Using HS compared to ½ MS medium accelerated flowering time by 3.5 d and flowering and seed setting rates significantly increased, by 21.2 and 11.2%, respectively (Table 4, *C2*). Compared to agar, perlite significantly increased flowering and seed setting rates by 18.4 and 19.1%, respectively, and resulted in an average of 4.7 fewer days to flower initiation (Table 4, *C3*). Furthermore, the supplementation with 4-Cl-IAA (0.05 μM) resulted in a higher flowering and seed setting rates (21.1 and 51.1%, respectively) and also accelerated flowering time by 7.2 d compared to IAA (5.7 μM) in the same substrate/nutrient solution combinations (Table 4, *C4*). The best combination of treatments among media, substrates, and plant growth regulators, in terms of higher flowering and seed setting rates, was the combination of HS with 0.05 μM 4-Cl-IAA and 0.9 μM flurprimidol using perlite as a substrate. This combination decreased the time to flowering to 31.8 d for the two lentil cultivars (9.0 d less compared to other media, substrates, and plant growth regulators). Under these conditions, 94.3% of all plants flowered, and 81.2% of all plants set seeds (20.2 and 51.8% increase, respectively, compared with other media, substrates, and plant growth regulators; Table 4, *C8*). Overall, the hydroponic method had a significantly positive effect on reproduction compared to tissue culture and intermediate methods (Table 4, *C9*, *C10*, *C11*).

Immature embryos were removed from pods approximately 12–14 d after anthesis (DAA) and thus one generation cycle was about 46 d (Figs. 2, 3). Taken together, the combination of perlite and HS nutrient solution plus 0.05 μM 4-Cl-IAA and 0.9 μM flurprimidol resulted in an average time to flower of 32 d, with a 94% flowering rate and a 81% seed setting rate (mean of the two cultivars). Therefore, it may be possible to produce eight generations of lentil within 1 yr using this approach. In contrast, only three generations per year are possible in the growth chamber or greenhouse using the conventional single seed descent (SSD) method. For SSD in self-pollinated pulse crops, it takes more than 2 yr to reach near-homozygosity and genetic stability. The RGT method produces about double the number of generations per year,

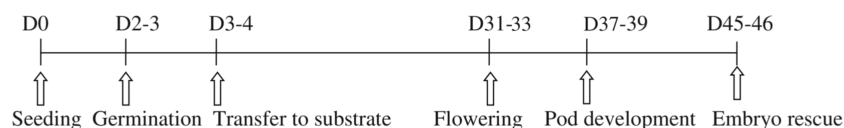

Figure 2. Average timelines in d for lentil generation cycle using rapid generation technology.

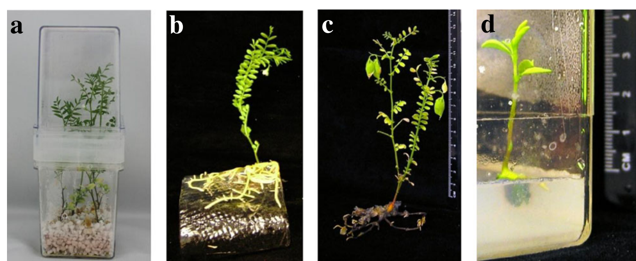

Figure 3. *In vitro* flowering of lentil (cv. 'CDC Maxim') with 0.05 μM 4-Cl-IAA + 0.9 μM flurprimidol in perlite (*A*) and agar (*B*), with four pods (*C*). (*D*) Regenerated plant 1 wk after embryo rescue.

and therefore has potential for significant acceleration of pulse crop breeding programs.

Effect of flurprimidol on growth of faba bean in vitro. The effect of different concentrations of flurprimidol was determined for vegetative and reproductive characteristics of two cultivars of faba bean. Initially, 0.3, 0.4, and 0.5 μM of flurprimidol were compared using a light intensity of 600 μmol m^{-2} s^{-1} with T5 cool white fluorescent tubes, Philips 841 (red/far-red=5.3) and 5.7 μM IAA in ChM1 medium. The results showed that 0.3, 0.4, and 0.5 μM of flurprimidol resulted in an average plant height of 9.7± 0.2 cm, with 10–13 nodes (data not shown). Days to flowering was reduced by 2.2 d (to 28.9 d) with a 52.6% increase in flowering rate using 0.3 μM flurprimidol compared to the higher concentrations of flurprimidol (Table 5, *C2*).

Effect of different combinations of auxin and zeatin on faba bean flower induction. The effect of different concentrations of *cis/trans* zeatin (0, 0.9, 2.3, or 4.5 μM) in combination with or without 5.7 μM IAA and with 0.3 μM of flurprimidol was determined for vegetative and reproductive traits of faba bean. The flowering rate was greatest (82%) when 5.7 μM IAA and 2.3 μM zeatin was added to the medium, while days to flowering were not significantly different with different levels of zeatin tested (Table 5, *C8*).

Subsequently, the effect of tissue culture (ChM1 medium with agar), hydroponic method (HS with perlite), and intermediate method (HS with agar) combined with two different auxins (IAA and 4-Cl-IAA) with or without zeatin was investigated for days to flowering and flowering and seed set rates. There was no significant difference between ChM1 *versus* HS, and agar *versus* perlite, for all three parameters (Table 6, *C1*, *C2*). Tissue culture, hydroponics, and intermediate methods were not significantly different among treatments (Table 6, *C8*, *C9*, *C10*); however, the combination of 5.7 μM IAA and 2.3 μM zeatin in combination with the intermediate method (HS with agar) led to 4.9 d earlier flowering and also increased flowering and seed setting rates by 28.4 and 56.0%, respectively, compared to all other treatments (Table 6, *C6*).

In most cases, zeatin combined with either IAA or 4-Cl-IAA increased flowering and seed setting rates of faba bean compared to IAA or 4-Cl-IAA alone (Table 6, *C4*, *C5*). The combination of the intermediate method with IAA (5.7 μM) and zeatin (2.3 μM) resulted in the highest rate of flowering (100%) and seed setting (91%) with the shortest flowering time (32 d).

Faba bean generation cycle. Since faba bean seeds are relatively large, the time required for germination of faba bean was greater than lentil, while seedling growth rate was faster. Under optimal conditions with the addition of PGRs (5.7 μM IAA + 2.3 μM zeatin + 0.3 μM flurprimidol) and use of HS medium, a mean of 32 d to flowering was achieved in both cultivars. The generation time was 54 d, including 18 d required for immature seeds to be ready for embryo rescue (Figs. 4, 5). Using *in vitro* flowering and RGT, 6.8 generations

Table 5. Effect of plant growth regulators on increase or decrease of faba bean *in vitro* flowering characteristics using orthogonal contrasts

Contrast	Plant growth regulator group	Plant height (cm)	No. of nodes	No. of branches	Days to flowering	Flowering rate (%)
C1	CDC FB 9-4 *vs.* CDC Snowdrop	1.9^{**}	2.7^{ns}	0.9^{ns}	1.8^{ns}	3.7^{ns}
C2	0.3 *vs.* 0.4 and 0.5 μM flurprimidol	1.8^{**}	0.8^{ns}	0.4^{ns}	-2.2^{**}	52.6^{***}
C3	0.4 ml *vs.* 0.3 and 0.5 μM flurprimidol	0.4^{ns}	-0.1^{ns}	-0.1^{ns}	-4^{**}	-19.4^{ns}
C4	0.5 ml *vs.* 0.3 and 0.4 μM flurprimidol	-2.2^{***}	-0.7^{ns}	-0.3^{ns}	6.1^{**}	-33.2^{***}
C5	5.7 μM IAA *vs.* 2.3 μM zeatin	2.5^{***}	1.3^{**}	0.1^{ns}	-4.6^{**}	40.9^{**}
C6	5.7 μM IAA *vs.* 5.7 μM IAA + 2.3 μM zeatin	1.2^{**}	0.4^{ns}	0.03^{ns}	-2.1^{**}	6.9^{ns}
C7	2.3 μM zeatin *vs.* 5.7 μM IAA + 2.3 μM zeatin	-1.4^{***}	-0.9^{**}	-0.06^{ns}	2.5^{**}	-34^{**}
C8	5.7 μM IAA + 2.3 μM zeatin *vs.* 5.7 μM IAA + 0.9 and 4.5 μM zeatin	1.7^{***}	0.6^{ns}	-0.4^{ns}	-0.7^{ns}	51.2^{**}

Data presented as delta value of means of cultivars CDC FB 9-4 and CDC Snowdrop

ns orthogonal contrast was not significant

**P*=0.05; ***P*=0.01; ****P*=0.001 (orthogonal contrast was significant)

Table 6. Effect of culture method on increase or decrease of faba bean *in vitro* flowering characteristics using orthogonal contrasts

Contrast	Culture method group	Days to flowering	Flowering rate (%)	Seed setting rate (%)
C1	ChM1 *vs.* HS	0.1^{ns}	6.8^{ns}	0.5^{ns}
C2	Agar *vs.* perlite	−0.1^{ns}	6.3^{ns}	2.5^{ns}
C3	0.05 μM 4-Cl-IAA *vs.* 5.7 μM IAA	−0.1^{ns}	15**	−6.5^{ns}
C4	5.7 μM IAA + 2.3 μM zeatin *vs.* 5.7 μM IAA	−2.2**	29.5**	48.9**
C5	0.05 μM 4-Cl-IAA + 2.3 μM zeatin *vs.* 0.05 μM 4-Cl-IAA	14.3***	63.3**	59.6***
C6	HS + agar + 5.7 μM IAA + 2.3 μM zeatin *vs.* other treatments	−4.9***	28.4**	56.0***
C7	HS + agar + 0.05 μM 4-Cl-IAA + 2.3 μM zeatin *vs.* other treatments	−2.4***	18.5*	21.4*
C8	Intermediate method *vs.* tissue culture method	−0.2^{ns}	−4.9^{ns}	1^{ns}
C9	Hydroponic method *vs.* intermediate method	0.3^{ns}	−3.9^{ns}	−3^{ns}
C10	Hydroponics method *vs.* tissue culture method	0.02^{ns}	−8.7^{ns}	−2^{ns}

Data presented as delta value of means of cultivars CDC FB 9-4 and CDC Snowdrop. All contrasts included medium with 0.3 μM flurprimidol

ns orthogonal contrast was not significant

$^*P$=0.05; $^{**}P$=0.01; $^{***}P$=0.001 (orthogonal contrast was significant)

per year could potentially be produced for faba bean, compared to 1 generation per yr in the field and three in the greenhouse.

Discussion

Rapid generation technology can reduce the time required to develop new varieties by reducing the time required to complete generation cycles. In some environments with long generation times, development of homozygous lines from segregating populations following hybridization takes 7–9 yr if only one generation is produced per year. Plant breeders have used various methods to accelerate generation turnover. For lentil and faba bean, efficient breeding programs produce two to three generations per year: one in the field during the crop season and the second and third in the off-season either in a greenhouse, growth chamber, or contra-season nursery. The RGT protocol accelerates generation turnover and has the potential to produce up to 6.8 generations per year for faba bean and 8 generations per year for lentil. These results are supported by those of Ochatt *et al.* (2002) and Ribalta *et al.* (2014), who demonstrated accelerated flowering and regeneration advancement in different genotypes of pea.

Flurprimidol reduces internode elongation through the inhibition of gibberellic acid biosynthesis (Ribalta *et al.* 2014), thus reducing plant height, which is an essential requirement for *in vitro* culture. The results of the current research, which has been illustrated in Fig. 1 for lentil and Table 5 for faba bean, are consistent with studies by Ochatt *et al.* (2002) and Ribalta *et al.* (2014) on pea in terms of ensuring adjustment of plant height to be optimal for culture vessel size by using flurprimidol. For example, in one of the pea genotypes, the highest concentration of flurprimidol reduced plant height from 77 cm (control treatment) into 13 cm. Flurprimidol decreased internode length *in vitro*; however, it did not affect number of nodes, flowering time, or seed set under both *in vivo* and *in vitro* conditions.

Induction of *in vitro* flowering and tomato fruiting was shown by Mamidala and Nanna (2009) to be related to the level of growth regulators added to the medium, as well as the influence of sugars and minerals. The authors reported that high rates of *in vitro* flowering and fruiting were observed using the combination of IAA (0.57 μM) and zeatin (9.1 μM) in tomato. *In vitro* rapid generation of velvet bean was investigated by Faisal *et al.* 2006. Three different cytokinins as supplements in half-strength MS medium were evaluated. Combination of cytokinin and auxin produced the greatest number of shoots with maximum shoot length. Also, optimal concentration of indole-3-butyric acid (IBA) led to best velvet bean root induction (Faisal *et al.* 2006). Rapid regeneration of embryogenic soybean accelerated cotyledon-stage embryos by 4 wk using liquid medium compared to solid MS medium which took 8 wk (Samoylov *et al.* 1998).

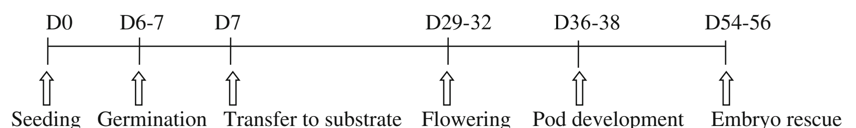

Figure 4. Average timelines for faba bean generation cycle using rapid generation technology.

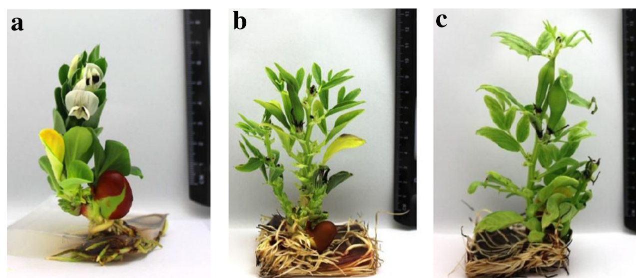

Figure 5. *In vitro* flowering of faba bean (cv. CDC FB9-4) using (*A*) 5.7 μM IAA + 0.3 μM flurprimidol, (*B*) 0.05 μM 4-Cl-IAA + 0.3 μM flurprimidol, and (*C*) 0.05 μM 4-Cl-IAA + 0.3 μM flurprimidol with 2.3 μM zeatin in agar substrate.

The benefit of PGRs for *in vitro* regeneration has been demonstrated in several rapid generation studies; however, few reports exist regarding the practical development of rapid generation techniques. In the current study, lentil flowering and seed setting rates were markedly improved by adding flurprimidol plus 4-Cl-IAA, whereas use of zeatin resulted in delayed flowering, combined with reduction of flowering and seed setting rate. The rate was further improved by defining the most effective substrate conditions for high seed setting rate in lentil, which was the hydroponic method (HS and perlite). Furthermore, this protocol resulted in a very high percentage of plants setting seed (>90%), which is critical for efficient development of recombinant inbred lines, and for maintenance of genetic diversity in populations.

Early flowering of faba bean under *in vitro* conditions in this study was achieved by establishing miniature plants through the use of flurprimidol (0.3 μM Topflor), IAA (5.7 μM), and zeatin (2.3 μM) using an intermediate culture method of hydroponics solution with agar as the substrate. This resulted in >90% of plants setting seed.

This is the first report on developing a rapid generation technique for lentil and faba bean. Since there is no published double-haploid protocol for these species, plant breeders need another method for quickly advancing generations.

Conclusions

Taken together, the combination of 0.9 μM flurprimidol and 0.05 μM 4-Cl-IAA was optimal for improving lentil flowering characteristics *in vitro*. For faba bean, the seed setting rate was more than 90% when 5.7 μM IAA, 2.3 μM zeatin, and 0.3 μM flurprimidol were applied. Overall, the hydroponic method is recommended for both lentil and faba bean, due to its cost-effectiveness and ease of operation and maintenance. The protocols developed in the present investigation may significantly contribute to genetic improvement of pulse crops *via* acceleration of flowering time, which promotes regeneration cycle and RIL development. Commercial hydroponic systems and perlite are lower in cost and require less intensive labor for utilization *in vitro* than MS or ChM1 and other agar-solidified media, thus simplifying the RGT protocol. The hydroponic solution can be further modified based on the plant growth stage to provide optimal nutrients for seed development. Future research will focus on developing *ex vitro* methods to further simplify and reduce the cost of RGT techniques.

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