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In Vitro Flowering Studies with Nine Cultivars of Perennial Ryegrass (*Lolium perenne* L.)

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ABSTRACT

We report here *in vitro* flowering in six cultivars namely; 'Banquet', 'Gulf annual', 'Linn Perennial', 'Meridian', 'Quartet' and 'Tolosa' of perennial ryegrass (*Lolium perenne* L.). The frequency of *in vitro* flowering varied from 2.5% for 'Tolosa' to 90% for 'Gulf annual'. Out of the five media (RM1-RM5) tested to induce flowering *in vitro*, only RM5 medium (MS salt + vitamin + 0.50 mg L^{-1} 6-benzyladenine (BA) + 2.0 mg L^{-1} thidiazuron (TDZ) + 38.0 mg L^{-1} $CuSO_4.H_2O$) produced *in vitro* flowering. Vernalization (one week at 4°C) of the *in vitro* shoots improved the flowering efficiency by 35% compared to the non-vernalized control. Callus was induced from mature seeds on medium containing MS salts and vitamins with 4.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 30 g L^{-1} sucrose. Unlike previously published reports of callus-based regeneration that requires several months in culture and associated somaclonal variation and albino shoot formation, we obtained normal shoots from mature seed-derived callus in 10-12 weeks.

Keywords: *in vitro* breeding, pasture grass, tissue culture, turf grass
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **BA**, 6-benzyladenine; **CIM**, callus induction medium; **GM**, genetically modified; **Kn**, kinetin; **PBZ**, paclobutrazol; **RM**, regeneration medium; **TDZ**, thidiazuron

INTRODUCTION

Ryegrass is the most widely cultivated forage grass in the world. Major species and hybrids of this genus include *Lolium perenne* L., *L. multiflorum* Lam., and *L. x boucheanum* Kunth. It is a nutritious and palatable forage grass with good adaptability and growth habits. Therefore, it is a preferred pastoral grass in North America, Europe, Australia, New Zealand and other temperate regions of the world (Jauhar1993; Harper *et al*. 2011; Sampoux *et al*. 2011).

Traditional breeding and biotechnology procedures have been applied to achieve genetic improvement of ryegrass, resulting in the release of improved cultivars (Yamada *et al*. 2005; Wang and Yamada 2008; Ghesquiere *et al*. 2010; Harper *et al*. 2011). Resistance to rusts, was improved at a rate of +11.39% per decade in the past few decades and dry matter yield was improved at a rate of 3.18% per decade (Sampoux *et al*. 2011). Traditional breeding in ryegrass takes a long time due to the fact that it flowers in 12-18 months and typically sets seeds after a brief vernalization treatment (Jauhar 1993; Reheul and Ghesquiere 1996). Another major challenge with improving *L. perenne* through conventional breeding is its self-incompatibility (Yamada *et al*. 2005; Yang *et al*. 2009). Additionally, the susceptibility of *L. perenne* cultivars to biotic stress (diseases) and abiotic stress such as cold, drought and salinity in the face of global climate change is a major challenge for ryegrass breeders developing novel cultivars (Yamada *et al*. 2005; Kosmala *et al*. 2007). Indirect regeneration of shoots through a callus and cell culture phases after prolonged periods of *in vitro* culture of ryegrass has been reported (Creemers-Molenaar *et al*. 1989, 1992; Dalton *et al*. 1988; Altpeter and Posselt 2000; Bradley *et al*. 2001; Newell and Gray 2005, Liu *et al*. 2006). Regeneration from cell culture derived protoplast cultures of ryegrass using conditioned medium is also reported (Folling *et al.* 1995). Polyploids, mixoploids and chimeras were among a group of *in vitro* regenerated rye grass plants after several months in culture (Creemers-Molenaar *et al*. 1992). Newell and Gray (2005) reported indirect regeneration of ryegrass plants from leaf base explants. Long period of incubation under *in vitro* (≥ 6 months) conditions resulted in somaclonal variation and albino shoot formation (Altpeter and Posselt 2000). Likewise, the effect of genotype on callus and shoot regeneration, rapid loss of regenerability from callus are some of the challenges for rapid cloning of ryegrass through tissue culture (Liu *et al*. 2006). Therefore, developing a protocol that allows rapid regeneration of callus and shoots *in vitro* with little or no genotype effect on regeneration and greater fidelity is highly desirable. Such an efficient and rapid regeneration protocol will improve the recovery of genetically stable transgenic ryegrass plants with greater efficiency. Genetic transformation of ryegrass with reporter genes using microprojectile bombardment (Spangenberg *et al.* 1995, 1998) and *Agrobacterium*-mediated genetic trans-formation (Bhalla *et al*. 1999; Altpeter *et al*. 2000; Altpeter 2006; Bajaj *et al*. 2006) have been reported. More recently, value added genes have been incorporated to develop salt resistant (Wu *et al*. 2005) and drought resistant (Zhao *et al.* 2007) transgenic ryegrass plants

In vitro flowering and seed set (*in vitro* breeding) can complement and advance the gains made through conventional and biotechnology mediated genetic improvements of ryegrass. Many plants flower faster *in vitro* than *ex vitro* thus the life cycle of the plant and generation time could be cut down with *in vitro* breeding strategy as demonstrated by Ochatt and Sangwan (2008) in *Arabidopsis thaliana. In vitro* flowering of bamboo, a monocarpic species with a long pre-bearing period was developed to reduce its breeding cycle (John and Nadgauda 1998). Sairam and Goldman (2009) also have discussed possible applications of *in vitro* flowering technology such as; reducing the breeding cycle, eliminating seasonality of breeding during adverse climate, rapidly advancing traits through breeding and/or transgenic seed production. Therefore, the current study was directed at developing an *in vitro* flowering system for ryegrass cul-

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tivars to advance traits through *in vitro* breeding that could accelerate breeding process for release of commercial cultivars. Rapid *in vitro* regeneration of shoots from mature seed-derived callus in nine cultivars of ryegrass with no albino shoot development was also achieved in this study.

MATERIALS AND METHODS

Plant material

Mature seeds of ryegrass cv. 'Bulldog', 'Gulf annual' and 'Linn perennial', commonly cultivated in the USA, as well as New Zealand cultivars of ryegrass cv. 'Bronsyn', 'Quartet', 'Impact', 'Banquet', 'Meridian' and 'Tolosa', were kindly supplied by Pastoral Genomics Ltd., New Zealand.

Seed germination and callus induction

Ryegrass seeds were washed thoroughly for 10 min with Tween-20®. The clean seeds were further surface sterilized following four different protocols (S1-S4). In Protocol S1, detergent washed seeds were treated with 50% (v/v) H_2SO_4 for 30 min. The acid treated seeds were left under tap water for two hours and transferred to a 56°C water bath for 15 min. The hot water treatment was used to kill the endophytic fungus (*Neotyphodium lolii*) known to harbor in ryegrass seeds following the report of Bajaj *et al.* (2006). The seeds were further treated with with 10% (v/v) Clorox® [Clorox Co., Oakland, CA, USA] solution for 15 min followed by three rinses with sterile distilled water to complete surface sterilization. In protocol S2, detergent washed seeds were treated with 90% (v/v) ethanol for two min followed by a 15 min treatment with 10% (v/v) Clorox®. In protocol S3, the concentration of bleach was increased to 25%. In protocol S4, the Clorox® treatment was replaced with a 0.1% (w/v) mercuric chloride treatment for 5 min following a 2-min treatment with 90% ethanol. Following the disinfection treatments, seeds were rinsed 5 times with sterile distilled water to complete the surface sterilization process. Surface sterilized seeds were cultured on autoclaved (121°C for 20 min at 1.2 kg cm^{-2} pressure) callus induction medium (CIM) [MS medium (Murashige and Skoog 1962) supplemented with 4.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L^{-1} sucrose and gelled with 7.0 g L^{-1} agar] in 10.0 × 100.0 mm polystyrene Petri dishes (VWR International, LLC, West Chester, PA, USA). At least 10 plates with 20 seeds/cultivar were used for seed germination and callus induction studies. The cultures were incubated in the dark at 25 ± 2°C for 2 weeks to achieve germination and callus induction. The seedling meristems were isolated from the germinated seeds along with the callus, subcultured on to fresh CIM and incubated under dark conditions for 4-6 weeks for further growth of callus. The calli were subcultured on to fresh CIM every two weeks to enhance callus growth.

Shoot regeneration from callus and induction of *in vitro* flowering

Approximately 0.5 cm pieces of rapidly proliferating, friable calli were transferred to tissue culture jars (14 cm × 6.0 cm) containing regeneration media (RM1-RM5). RM1 consisted of MS salts and vitamins plus 0.5 mg L^{-1} 6-benzyladenine (BA), 0.1 mg L^{-1} 2,4-D, 38.00 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 30g L^{-1} sucrose and 7.0 g L^{-1} agar. The media composition of RM2-RM5 was:

RM2: MS + vitamins + 0.5 mg L^{-1} BA + 0.10 mg L^{-1} 2,4-D + 38.0 mg L^{-1} $CuSO_4 \cdot 5H_2O$ + 1.0 mg L^{-1} paclobutrazol (PBZ);

RM3: MS + vitamins + 0.5 mg L^{-1} BA + 0.10 mg L^{-1} 2,4-D + 38.0 mg L^{-1} $CuSO_4 \cdot 5H_2O$ + 2.0 mg L^{-1} PBZ;

RM4: MS + vitamins + 0.5 mg L^{-1} BA + 0.10 mg L^{-1} 2,4-D + 38.0 mg L^{-1} $CuSO_4 \cdot 5H_2O$ + 5.0 mg L^{-1} PBZ;

RM5: MS + B5 vitamins (Gamborg *et al*. 1968) + 0.5 mg L^{-1} BA + 2.0 mg L^{-1} thidiazuron (TDZ) + 38.0 mg L^{-1} $CuSO_4 \cdot 5H_2O$.

These cultures were incubated at 25 ± 2°C in the light at irradiance of 30 µmol m^{-2} s^{-1} provided by cool, white, fluorescent tubes (Philips, USA - http://www.advance.philips.com) set at a 16-h photoperiod for 4-6 weeks to achieve shoot regeneration. The regenerating calli were subcultured on to fresh RM1-RM5 in tissue culture jars and incubated for 4 weeks to regenerate shoots. *In vivo* flowering of perennial ryegrass requires vernalization and is also known to be under photoperiodic control (Skøt *et al*. 2011). Therefore, the shoot clumps were vernalized at 4°C for 7.0 days and exposed to long day conditions (16 h light) to induce *in vitro* flowering.

RESULTS AND DISCUSSION

Surface sterilization and seed germination

Four protocols were tested for surface sterilization of seeds. Among the four, S2 and S3 yielded good germination rates. The highest percentage seed germination (93%) was recorded by 'Banquet' following protocol S3 followed by 'Linn perennial' (87.5%) following protocol S2. The New Zealand cultivar 'Bronsyn' recorded the lowest rate of seed germination (22%) when decontaminated using protocol S3 (**Table 1**). Protocol S3 was rated best for surface sterilization because it offered excellent control of contamination while promoting good seed germination in most of the cultivars studied. Lower germination rates (e.g. 'Impact' and 'Banquet') using protocol S2 in comparison with S3 can be attributed to some of the seed contamination as seen in **Table 1**. Seed germination rates can vary between the seed lots and seeds of different cultivars for a number of reasons such as maturity at harvest, dormancy, inherent sterility, age of the seed lots, steps in the processing of seed lots, etc. (Sairam *et al*. 2002). It is well reported in literature, seed germination can be hampered by disinfectants such as alcohol, chlorine and mercuric chloride because of their harmful effects on living cells. The variation in the contamination rates observed between the varieties could be a result of the type of contaminants (different species of bacteria and fungi) present in the seed lots and their relative susceptibility to the disinfectants used. The material safety data sheet (http://msds.chem.ox.ac.uk/ME/mercury_II_chloride.html) for mermercuric chloride indicates that it inhibits the respiratory pathway, which partially explains the observed lower germination of seeds treated with $HgCl_2$ compared to milder disinfectants like bleach and alcohol. Sulfuric acid (http://msds.chem.ox.ac.uk/SU/sulfuric_acid_concentrated.html) is a strong, corrosive and toxic acid, and hence the treatment with 50% H_2SO_4 for 30 min may have killed the seeds by damaging the embryos.

Callus induction

All the ryegrass genotypes studied germinated and produced callus in CIM. Callus induction frequency across all genotypes tested was 100%. The root-shoot juncture was the best explant for callus induction. Previous tissue culture studies with mature seed explants of ryegrass (Bajaj *et al*. 2006; Liu *et al*. 2006) also showed callus induction in MS medium supplemented with sucrose and 2,4-D. Similarly, MS medium supplemented with sucrose and 2,4-D at low concentrations (1.0-5.0 mg L^{-1}) have been shown to generate callus in many members of the Poaceae family [e.g., maize (Sairam *et al*. 2003) rice (Chauhan *et al*. 1998; Amarasinghe 2009), sorghum (Sairam *et al*. 2000); Tripsacum (Sairam *et al*. 2002); sweet sorghum (Sadia *et al*. 2010)]. Based on our experience with successful tissue culture of various grasses in our lab as cited above, we designed the germination and callus induction media to suit all nine genotypes of ryegrass included in this study. Similarly, the observation that the root-shoot juncture is the best source of callus production is consistent with other reports on ryegrass tissue culture (Bradley *et al*. 2001; Bajaj *et al*. 2006; Liu *et al*. 2006) as well as results of our studies with grass species like maize, rice, sorghum and sweet sorghum (Chauhan *et al*. 1998; Sairam *et al*. 2000, 2003; Sadia *et al*.

Table 1 Effect of surface sterilization on seed germination in different varieties of ryegrass.

Cultivar	Percent regeneration following different surface sterilization procedures			
	S1	**S2**	**S3**	**S4**
Gulf Annual	0	49/90 (54.4%)	122/220 (55.5%)	129/220 (58.6%)
Bulldog	1/220 (0.45%)	134/210 (63.8%)	103/220 (46.8%)	80/220 (36.36%)
Linn Perennial	0	79/90 (87.8%)	172/220 (78.2%)	124/220 (56.4%)
Bronsyn	not done	15/80 (18.8%)	22/100 (22.0%)	43/220 (19.54%)
Quartet	not done	146/177 (82.5%)	50/100 (50.0%)	73/220 (33.18%)
Impact	not done	20/150 (13.3%)	45/100 (45.0%)	8/100 (8.0%)
Banquet	not done	79/200 (39.5%)	93/100 (93.0%)	39/100 (39.0%)
Meridian	not done	90/200 (45.0%)	81/100 (81.0%)	72/100 (72.0%)
Tolosa	not done	16/200 (8.0%)	104/200 (52.0%)	42/100 (42.0%)

S1 = 30 min 50% H_2SO_4 - 2 h running water -10 min 10% bleach, S2 = 2 min 90% ethanol – 10 min 10% bleach, S3 = 2 min 90% ethanol – 10 min 25% bleach, S4 = 2 min 90% ethanol – 10 min 25% bleach – 5 min 0.1% $HgCl_2$.

Table 2 Percent regeneration of shoots from ryegrass calli cultured on RM5 medium for and subsequent flowering *in vitro*.*

Cultivar	# of Jars	#of Calli	# Regenerated	% Regeneration	# Flowered	% Flowering
BD	18	82	82	100 a	0	0 e
GA	20	96	96	100 a	86	89.58 a
LP	13	60	52	90 b	21	40.38 b
BR	17	77	69	89.61 bc	0	0 e
QU	25	123	120	97.56 b	39	33.5b c
IM	14	67	62	92.5 b	0	0 e
BA	20	102	88	86.27 bc	22	25 c
ME	15	76	60	78.94 c	3	5 d
TO	20	98	82	83.67 bc	2	2.4 d

*Calli bearing shoot clumps were vernalized for a week at 7.0 days at 4°C for 7.0 days, subsequently transferred to RM5 medium and incubated under 16 h light for 30 days to induce *in vitro* flowering. RM5: MS + B5 Vitamin + 0.5 mgL^{-1} BA + 2.0 mgL^{-1} TDZ + 0.1 mg L^{-1} 2,4-D, + 38.0 mgL^{-1} $CuSO_4 \cdot 5H_2O$, pH 5.8. BD: Bull dog, GA: Gulf annual, LP: Linn perennial, BR: Bronsyn, QU: Quartet, IM: Impact, BA: Banquet, ME Meridian, TO: Tolosa. n =13 or more, values with different letters in a column indicate significant differences according to Student's t-test ($P < 0.05$).

2010).

Shoot regeneration and *in vitro* flowering

Five different media were tested for shoot regeneration from callus. Two media in particular, RM1 and RM5, were tested on all nine varieties. The regeneration frequency on RM1 varied from 35.82% in 'Banquet' to 65.63% in 'Quartet'. In comparison, the regeneration frequency on RM5 varied from 78.94% in 'Meridian' to 97.56% in 'Quartet'. Based on the observation that RM5 is the best medium for shoot proliferation and growth, we only used the RM5 medium for further regeneration and flowering experiments (**Table 2, Fig. 1A-D**). The callus cultures with shoot clumps were vernalized prior to their incubation on RM2-RM5 media. Therefore, we suspect that in addition to the high concentration and combinations of cytokinins in RM5, vernalization also played a key role in the enhanced rate of shoot regeneration. Concordantly, vernalization has been shown to enhance regeneration of winter wheat shoots derived from immature inflorescence cultures (Kavas *et al*. 2008). Likewise the rate of regeneration and number of shoots produced per cultured callus tissue varied among the varieties studied (data not shown). This variation in the regeneration rate and frequency of shoot multiplication could also be a result of the genotypic difference among the varieties studied. Liu *et al*. (2006) reported similar variation in shoot regeneration frequency for ryegrass (from 2.8% for 'Barclay' to 38.8% for 'Barlennium'). Similarly, genotype effect on *in vitro* development of barley (Castillo *et al*. 1998), tall fescue (Bai and Qu 2000) and sweet sorghum (Sadia *et al*. 2010) have been reported. Elevated levels of cupric sulfate have been shown to enhance shoot regeneration *in vitro* from callus cultures of cereals such as barley (Wojnarowiez *et al*. 2002), rice (Amarasinghe 2009), and sorghum (Nirwan and Kothari 2003). Shoot regeneration from callus cultures of *Panicum virgatum* was also enhanced by 38.0 g L^{-1} copper sulfate (unpublished data). Copper is an important micronutrient for plants as it plays an important role in CO_2 assimilation and ATP synthesis (Thomas *et al*. 1998). Copper is also important for plants as an essential component of protein plastocyanin and cytochrome oxidase enzyme in the respiratory pathway (Demirevska-Kepova *et al*. 2004).

As discussed earlier, five modifications of MS medium (RM1-RM5) were tested for their ability to induce flowering *in vitro*. Although shoots regenerated and grew to 4-6 cm in different RM media studied, *in vitro* flowering could only be obtained in the RM5 medium (**Fig. 2A, 2B**). In addition to the incubation on auxin free MS medium supplemented with a combination of TDZ (2.0 mg L^{-1}) and BA (0.5 mg L^{-1}), vernalization of the shoots at 4°C for 7 days improved *in vitro* flowering. However, in this study medium supplemented with a combination of BA and PBZ at 1-5 mg L^{-1} (RM2-RM4) did not induce flowering *in vitro*. Different ryegrass cultivars exhibited *in vitro* flowering to different extents: 'Gulf annual' (90%), 'Linn perennial' (40%), 'Quartet' (33.5%), 'Banquet' (25%), 'Meridian' (5%) and 'Tolosa' (2-5%) in this study.

Natural flowering is a complex physiological process (Boss *et al*. 2004) that is induced mainly by day length and temperature cues from nature. These natural cues such as day length can cause changes in the hormonal regime of the plants, leading to the termination of vegetative growth of the plant to the reproductive structures. Excellent reviews on regulation of flowering have been reported earlier (Bernier and Perilleux 2005; Heggie and Halliday 2005). Specifically four pathways have been reported in *Arabidopsis* namely; autonomous, photoperiod, gibberellin and vernalization (Mouradov *et al*. 2002; Koornneef *et al*. 1998; Simpson and Dean 2002) induced flowering.

In vitro flowering has been reported in many monocot and dicot plants such as bamboo (John and Nadgauda 1999; Lin *et al*. 2003, 2004), corn (Mandal and Elanchezhian 2000) *Lilium* (Ishimori *et al*. 2009), switchgrass (Alexandrova *et al*. 1996), date palm (Masmoudi-Allouche *et al*. 2011), orchids (Ferreira *et al*. 2006; Sim *et al*. 2007), olive (Chaari-Rkhis *et al*. 2006), and tomato (Sheeja and Mandal 2003). However, to our knowledge, *in vitro* flowering has not been reported in *L. perenne*.

Components of the culture medium such as carbon source and hormones, light (quality, quantity and photoperiod) as well as incubation temperature and vernalization have been shown to affect *in vitro* flowering in different plant species. *Lolium perenne* normally flowers during the warm, long days in summer (Skøt *et al*. 2010). Therefore, the applied long day condition (16.0 h light) may have been a contributing factor of *in vitro* flowering. Components of

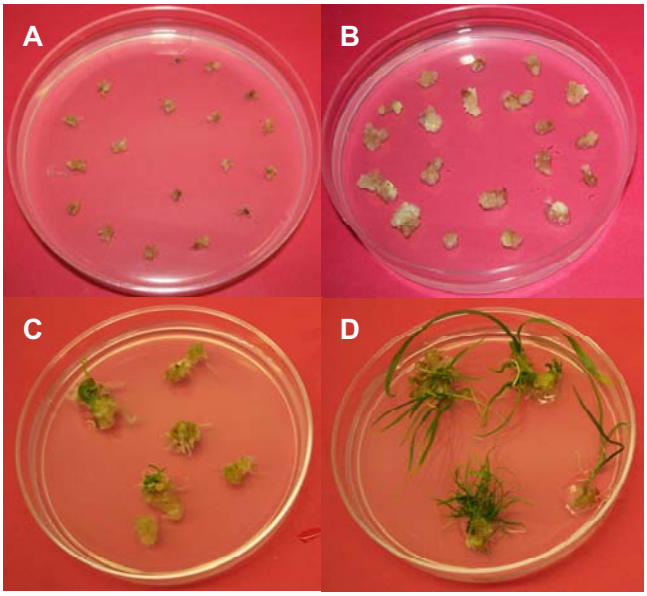

Fig. 1 Callus-based regeneration of ryegrass cv. 'Bull dog'. (**A**) Callus induction from seeds; (**B**) Proliferation of callus; (**C**) Regenerating calli; (**D**) Regenerated shoot clumps.

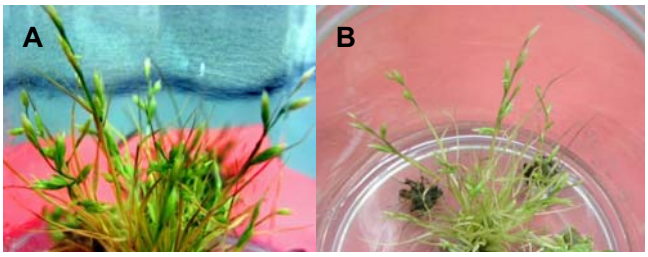

Fig. 2 *In vitro* flowering of ryegrass. *In vitro* flowering of ryegrass incubated on RM5 media following 7.0 day vernalization at 4°C. Photographed after 6.0 weeks' incubation in RM5. (**A**) 'Gulf annual' (**B**) 'Banquet'.

the culture medium (especially sucrose concentration), hormones (kinetin (Kn), BA, PBZ, GA_3, TDZ), and the photoperiod are key variables reported to influence flowering *in vitro*. Singh *et al*. (2006) reported 2-3 mg L^{-1} Kn and a 12-16 h photoperiod as important factors conducive to *in vitro* flowering in Kinnow mandarin. PBZ (0.25-0.50 mg L^{-1}) in the medium induced *in vitro* flowering in *Dendrobium* shoot cultures (Te-chato *et al*. 2009). Brito *et al*. (2003) reported that *Ceropegia bulbosa* cultures incubated on MS medium supplemented with 0.5 mg L^{-1} BA and 1.0 mg L^{-1} NAA exhibited *in vitro* flowering, but the same species produced only microtubers when maintained on MS medium containing kinetin and IBA. High levels of sucrose (30 g L^{-1}) in the medium favored *in vitro* flowering in mandarin (Singh *et al*. 2006), Gentiana (Zhang and Leung 2000), and bamboo (Lin *et al.* 2004). We have been maintaining the ryegrass shoot cultures on MS medium fortified with TDZ, BA and 30 g L^{-1} sucrose under a long day condition (16.0 h light) for four weeks to induce flowering. TDZ is known to enhance the cytokinin activity in many plants (Murthy *et al*. 1998), and cytokinins are known to induce *in vitro* flowering in plants as discussed earlier. Therefore, we assume that *in vitro* flower induction could have been a result of multiple factors such as vernalization, media composition (sucrose, TDZ and BA), as well as the incubation under long day conditions.

CONCLUSION AND FUTURE CONSIDERATIONS

In this study, we established efficient, callus based regeneration of nine ryegrass cultivars from mature seed explants and *in vitro* flowering of six cultivars. Perfecting a protocol for *in vitro* flowering and seed set of ryegrass to efficiently conduct *in vitro* breeding is important because it can reduce the breeding cycle, extend the growing season, allow breeding through usually unfavourable seasons (short winter days) and thus accelerate breeding to realize rapid genetic gains and development of new, improved cultivars. This non-GM, *in vitro* breeding method will also reduce the time needed to release improved cultivars to farmers.

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