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Research article

In vitro propagation of six parental lentil (*Lens culinaris* ssp. *culinaris*) genotypes

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Abstract

An efficient *in vitro* propagation method was developed for six lentil (*Lens culinaris* ssp. *culinaris*) genotypes: Digger (ILL5722), Indianhead, Nipper, Northfield (ILL5588), ILL 7537 and ILL 6002 that represent the genetic basis of the Australian lentil breeding program. Mature seeds were initially cultured on Murashige and Skoog (MS) medium supplemented with 4 mgL^{-1} of benzyladenine (BA). The maximum number of shoots per seed was 4.13±0.33. The best adventitious shoots proliferation rate (6.42±0.81 shoots per explant) was obtained from single shoots excised from two-week-old axenic germinated seedlings subcultured onto MS medium containing 3 mgL^{-1} BA. Roots of normal morphology were produced from up to 90 % of the *in vitro* regenerated nodes when transferred to MS medium supplemented with 4 mgL^{-1} α-naphthaleneacetic acid (NAA) and up to 80% of the regenerated plantlets were transferred to a soil environment with normal flowering and seed set.

Keywords: lentil, lens culinaris, micropropagation, multiple shoots, rooting

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Introduction

Lentil (*Lens culinaris* ssp. *culinaris*) is an annual cool season grain legume produced as a high protein food source throughout the world. In Australia, lentil has been grown as a commercial crop since the late 1980's and the seed is produced largely for export to south Asia and the Middle East. The breeding program is reliant on relatively few parental lines and is thus genetically narrow (Ford *et al.* 1997). The genotypes mainly used were; Northfield (ILL5588), Digger (ILL 5722), Nipper, ILL7537, and Indianhead, which contain resistances to important diseases

such as *Ascochyta lentis* (Ford *et al.* 1999; Chowdhury *et al.* 2001; Nguyen *et al.* 2001; Ye *et al.* 2003), and ILL 6002, which is a vigorous, tall, erect and early flowering variety used to develop new varieties with improved characteristics for easier and efficient harvesting (Materne, 2002).

Several studies have uncovered genomic regions and even specific genes responsible for governing desirable traits, for incorporation into breeding programs (Ford *et al.* 1999; Ta'ran *et al.* 2003). Molecular mapping efforts have identified markers that are close and flanking chromosomal loci within which the major trait-influencing genes are located and marker-assisted selection for improved disease resistance is underway in Australia and Canada (Muehlbauer *et al.* 2006). Microarray analysis has identified some of the functional genes that convey resistance to *Ascochyta lentis* in the highly resistant line ILL7537 (Mustafa *et al.* 2009). In order to speed up the breeding and selection of elite varieties, that contain the genes of interest, a transgenic approach may be undertaken. For this, a reliable and highly reproducible *in vitro* regeneration system, that is applicable to the targeted genotypes, is required. In addition an *in vitro* culture protocol would enable mass multiplication of high quality and disease-free clonal plants.

The development of an *in vitro* regeneration system for lentil has previously been studied using different tissues such as shoot apices (Bajaj & Dhanju 1979; Polanco *et al*. 1988; Polanco & Ruiz 1997; Singh & Raghuvanshi 1989; (Bajaj & Dhanju 1979; Polanco *et al*. 1988; Polanco & Ruiz 1997; Singh & Raghuvanshi 1989; Williams & McHughen 1986). According to Williams and McHughen (1986), calli derived from lentil shoot meristems and epicotyls regenerated shoots at a 50% rate. However, roots could not be induced on media containing either indole acetic acid (IAA) or naphthyl acetic acid (NAA), commonly used for root induction in plant tissue culture systems. Instead, only 11% of shoot explants developed roots when cultured on a sand-bed. Bractlets used by Polanco and Ruiz (1997) had a higher potential for root induction with a lower level of benzylaminopurine (BAP) in the media. In contrast, callus was induced when lentil bractlets were cultured on higher levels of BAP in the media (Polanco & Ruiz 1997).

Immature seed were also used as an explant source (Fratini & Ruiz 2006; Polanco & Ruiz 1997, 2001) and Polanco and Ruiz (2001) reported no significant differences on shoot regeneration rates from immature seed among different genotypes. In contrast, they did report differences between lentil genotypes on rates of root induction (Polanco and Ruiz 2001). Lentil seeds cultivated on NAA medium had the best rooting rate at 50%, and the rooted plants had 80-100% survival rate when hardened off in the glasshouse (Ye *et al*. 2002).

Cotyledonary nodes were also used as an explant source and assessed for regeneration on BAP-containing media (Cocu *et al*. 2003; Gulati *et al*. 2001; Sarker *et al*. 2003; Wakentin & McHughen 1993); however, there was no significant difference in the rooting rates among genotypes in Gulati's report. Other explant sources previously used have included nodal explants (Ahmad *et al*. 2006; Fratini & Ruiz 2008; Polanco *et al*. 1988; Singh & Raghuvanshi 1989; Ye *et al*. 2002), intact seedlings (Malik & Saxena 1992) as well as somatic embryogenesis (Saxena & King 1987). Generally, techniques to regenerate shoots have been successful. However, for the majority of previous studies, the ability to reproducibly regenerate roots from regenerated shoots has been far less successful and studies have contradicted (Altaf 2007). Among all of the explant sources assessed, cotyledonary nodes were optimal for multiple shoot formation. Regeneration methods also exist *via* somatic embryogenesis (Saxena and King 1987). However, not only are the successes of these methods very genotype-specific, there has been considerable difficulty reported in root production and hence whole plant regeneration.

Therefore, the aim of this study was to develop a reproducible *in vitro* regeneration protocol from cotyledonary nodes for the six important lentil genotypes; Northfield (ILL5588), Digger (ILL 5722), Nipper, ILL7537, Indianhead and ILL6002.

Materials and Methods

Plant material

Homozygous (inbred) seed of Northfield (ILL5588), Digger (ILL 5722), Nipper, ILL7537, Indianhead and ILL6002 were provided by the Australian Temperate Field Crop Collection, Horsham, Australia. They were surface sterilized

by immersion for 1 min in 70% ethanol followed by a 10 min wash in 10 % sodium hypochlorite (NaOCl) containing 0.01% (v/v) Tween 20, a 5 min wash in 5% NaOCl containing 0.01% (v/v) Tween 20 and three 5 min washes in sterile H_2O.

Effect of BA on in vitro seed germination

Sterilized seed were plated on MS (Murashige and Skoog) medium containing 30 gL^{-1} sucrose, 7 g/L^{-1} agar and 0, 1, 2, 3, or 4 mgL^{-1} benzyladenine (BA) (pH 5.8). Each treatment was replicated 10 times (one seed per culture tube) and the entire experiment was repeated trice. Cultures were maintained in a growth chamber at 25 ºC under a 16-h light/ 8-h dark photoperiod and the number of shoots per germinated seed and shoot length were counted after four weeks.

Effect of BA on multiple shoot production

Single shoot explants of 1 cm in length were excised from two-week-old axenic germinated seedlings. These were cultured on MS medium supplemented with 30 gL^{-1} sucrose, 7 gL^{-1} agar and 0, 1, 2, 3 or 4 mgL^{-1} BA (pH 5.8). Three explants were cultured per culture bottle and each treatment was repeated 10 times. Cultures were maintained in a growth chamber at 25 ºC under a 16-h light/ 8-h dark photoperiod and the number of shoots/per explant was counted after four weeks.

Effect of NAA on root induction

Single nodes of 1 cm in length were excised from two-week-old axenic germinated seedlings and cultured on MS medium supplemented with 30 gL^{-1} sucrose, 7 gL^{-1} agar and 0, 1, 2, 3 or 4 mgL^{-1} of α-naphthaleneacetic acid (NAA) (pH 5.8). One explant was placed in each culture tube and each treatment was repeated 10 times. Cultures were maintained in a growth chamber at 25 ºC under a 16-h light/ 8-h dark photoperiod and the percentage of root induction per treatment was determined after four weeks.

Hardening off and whole plant regeneration

All *in vitro* plantlets that produced a root-like structure were placed on MS medium supplemented with 30 gL^{-1} sucrose and 3.5 gL^{-1} agar to recover for 7 days. The roots of surviving plantlets were subsequently washed in sterile H_2O to remove agar and then placed in sterile soil medium (50% peat, 25% perlite, 15% vermiculite, 10% coarse sand, 50 mgL^{-1} dolomite to pH 7.0. Foliage was covered to maintain high humidity and placed in a controlled environment growth room at 20 ºC under a 16-h light/ 8-h dark photoperiod. After 5 days, plants were uncovered and removed to the glass house maintained at 20±2 ºC during the months of September-October, Melbourne, 2009 and those that proceeded to flowering and appeared morphologically normal were noted.

Results and Discussion

Effect of BA on in vitro seed germination

The number of shoots produced per germinated seed was generally not significantly different among genotypes and BA concentrations ($P<0.05$), although seed cultured without BA produced the lowest mean number of shoots at four weeks. Of the BA concentrations assessed, 4 mgL^{-1} induced the highest mean number of shoots per germinated seed with ILL7537 producing significantly higher shoot numbers (6.70±0.72 shoots). BA promotes cell division, shoot proliferation and shoot morphogenesis and has previously been reported to influence the formation of multiple shoots from a single seed in a Spanish lentil cultivar (Fratini and Ruiz, 2002). A similar pattern of BA-dependant variation in shoot production was also reported from seed of 10 inbred lines of four *Lens* species; *L. culinaris*, *L. nigricans*, *L. ervodides* and *L. odemensis* (Ye *et al.* 2002).

Although BA is useful for the purposes of producing multiple shoot explants from a single seed for future culture manipulation, this hormone has also been reported to inhibit shoot elongation in lentil (Fratini and Ruiz 2002; Ye *et al.* 2002). When seed of the six lentil genotypes in the current study were germinated on MS medium without BA, Nipper and ILL6002 produced shoots that were significantly shorter than those of the other four genotypes (4.11±0.93 cm and 3.65±0.91 cm, respectively; $P<0.05$). Subsequently, the shoot lengths of all genotypes assessed were significantly shortened by the addition of 1 mgL^{-1} of BA, with Nipper and ILL6002 producing the shortest shoots. However, increasing the BA concentration to 2, 3 or 4 mgL^{-1} did not cause any further significant decrease in shoot length.

Effect of BA on multiple shoot production

As previously shown from germinated seeds, the mean number of shoots that were multiplied from single excised shoot explants was significantly greater when BA was included in the media. However, of all treatments assessed, the only significantly responsive genotype was Digger, when placed on media containing 3 or 4 mgL^{-1} BA (19.92±2.07 and 12.00±2.49 shoots, respectively). This indicated a genotype-specific response to shoot multiplication as previously reported in other crop species (Zhang and Bhalla, 2004; Bhatia *et al.* 2005). BA cytokinin is well known to stimulate adventitious shoot formation and Polanco *et al.* (1988) previously obtained the optimal multiple shoot formation from shoot tips and nodes of three Spanish lentil cultivars on MS medium supplemented with 2.25 mgL^{-1} BA.

Effect of NAA on root induction

No root formation was observed on any of the six genotypes cultured on MS medium without the addition of NAA. Both Indianhead and IL7537 were the most responsive to NAA, with roots formed from single nodes grown on a concentration of 1 mgL^{-1} (30 and 40%, respectively). All of the other genotypes assessed required a NAA concentration of $\geq$2 mgL^{-1} to form a root structure. Of the concentrations assessed, 4 mgL^{-1} NAA was optimal to induce roots on all of the genotypes assessed. Polanco and Ruiz (2001) previously reported the stimulatory effect of NAA on root induction in lentil, with a rooting efficiency of 89% on 1 mgL^{-1} NAA. Similarly, Ye *et al.* (2002) determined that 1.5 mgL^{-1} NAA induced roots from >50 % of the shoots assessed from four lentil species.

Hardening off and whole plant regeneration

Of the surviving rooted plantlets, Indian head was most successful transferred and survived to flowering and seed set with normal morphology (mean of 80% across all treatments), Digger and Nipper were the next most successful (40%), then ILL6002 and ILL5588 (20%) with ILL7537 the least successful (10%).

In conclusion, although the effect of BA and NAA were somewhat genotype specific, an efficient and reliable *in vitro* propagation protocol was developed for each of the six genotypes paramount to the Australian lentil breeding program. This provides an efficient means for large scale propagation of cloned plantlets from seed culture and may be used in future lentil transgenic studies.

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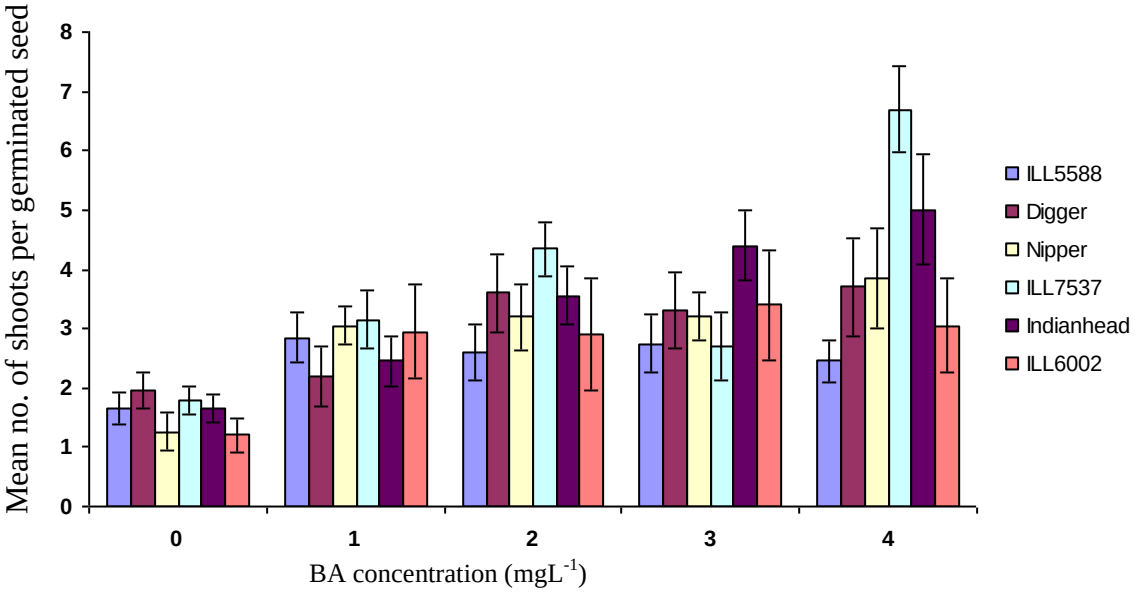

Figure 1: The shooting response of germinating seeds of six important lentil parental genotypes to BA concentration in MS medium.

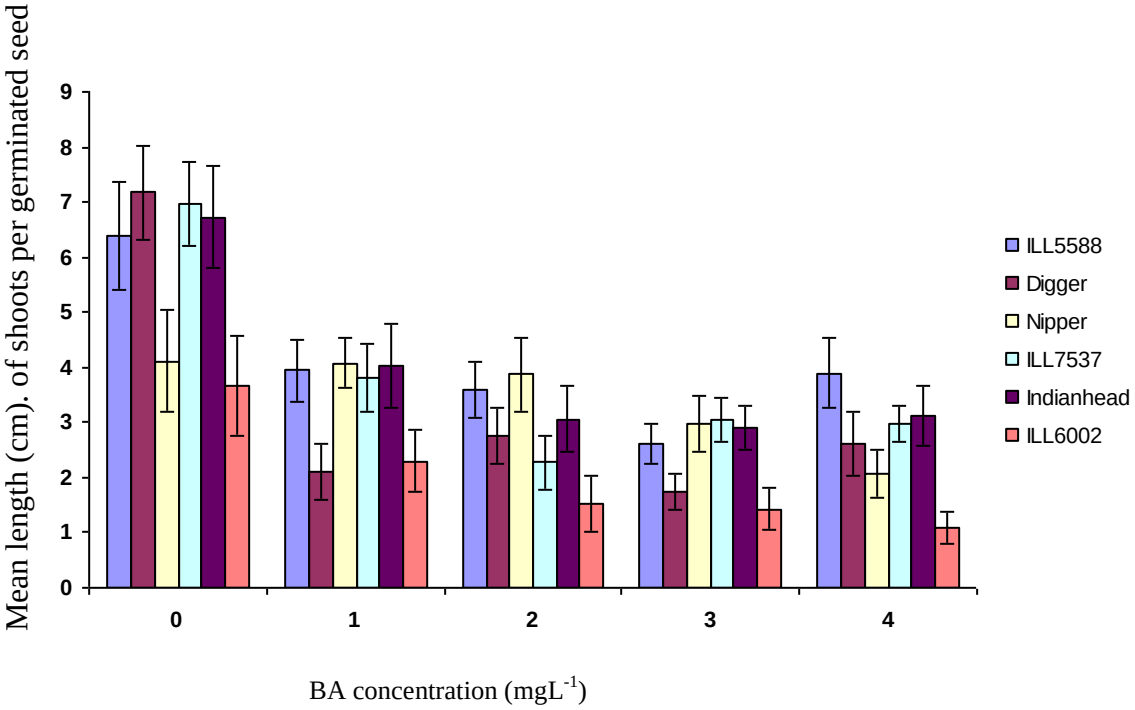

Figure 2: The mean length of shoots from germinating seeds of six important lentil parental genotypes on BA concentrations in MS medium.

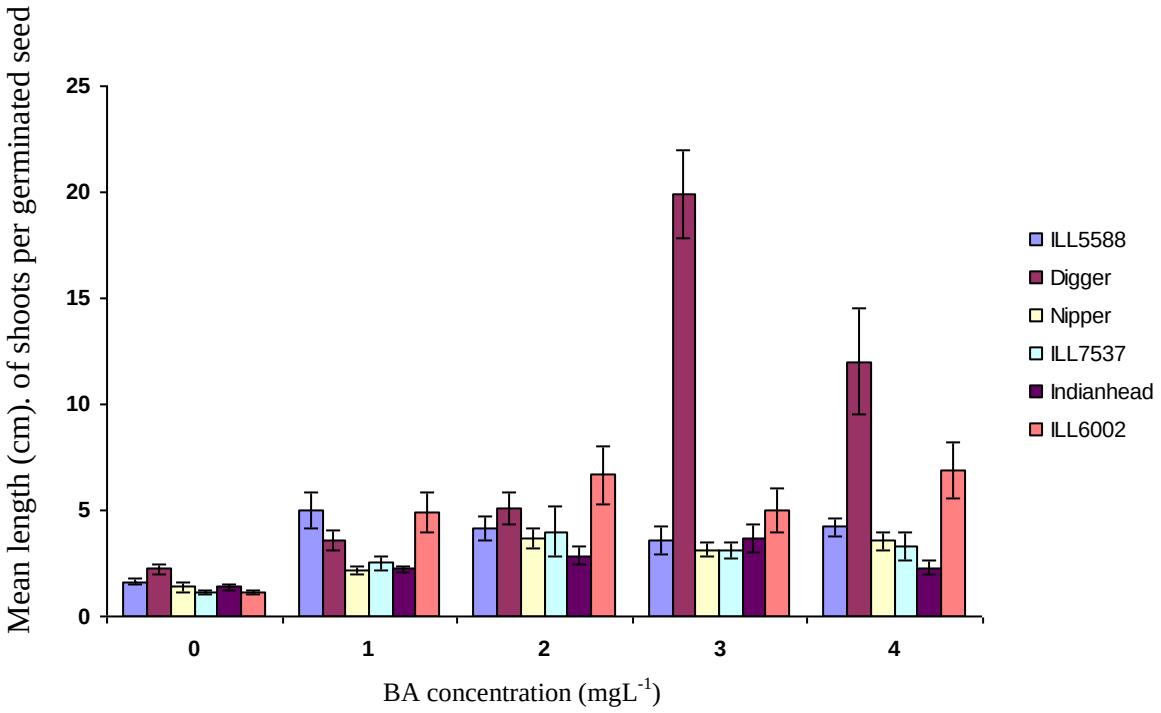

Figure 3: Mean number of shoots multiplied from a single shoot explant of six important lentil parental genotypes in response to BA concentration in MS medium.

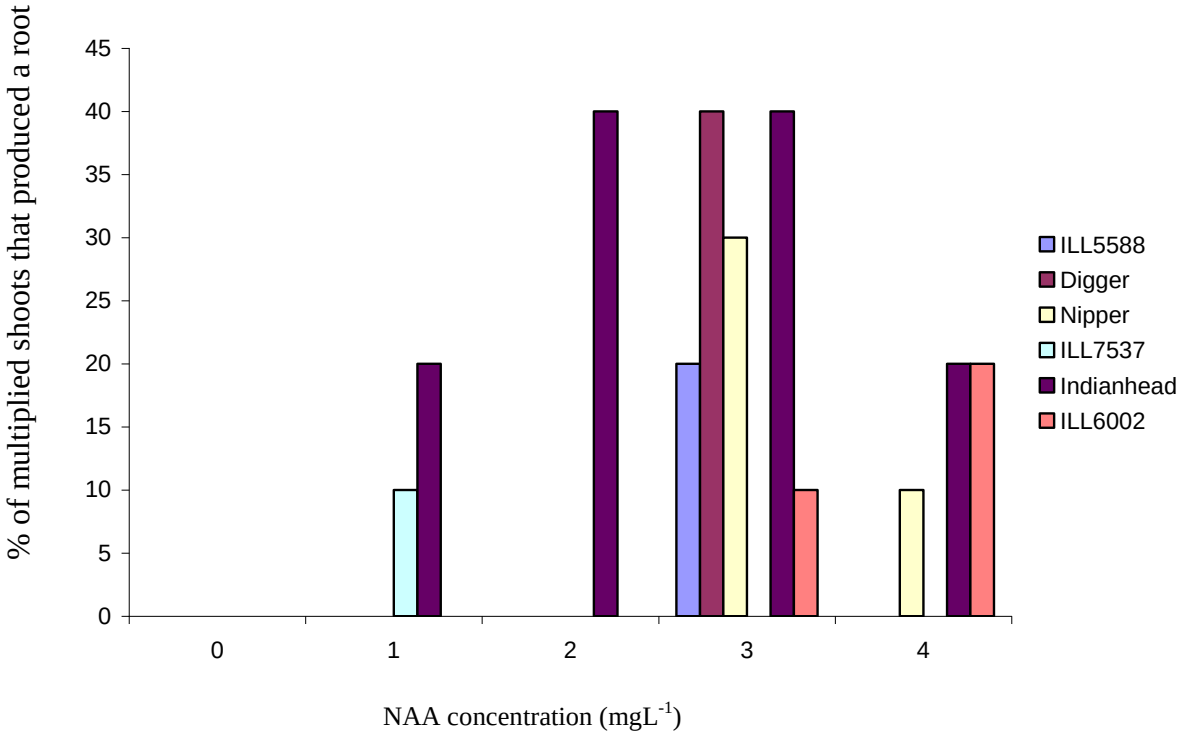

Figure 4: Percent of multiplied shoots that formed a root for six important lentil parental genotypes in response to NAA concentration in MS medium.