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In vitro flowering and seed formation in lentil (*Lens culinaris* Medik.)

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Introduction

Grain legumes, commonly known as pulses, are one of the most important agricultural crops worldwide. Pulses are amongst the ancient food crops that have been traditionally cultivated, particularly in Asia, Latin America, and Africa. In many developing nations, grain legumes are gaining increased importance in view of the acute shortage in production of animal proteins and widespread protein malnutrition (Bressani 1973). Apart from this, grain legumes are also considered as the main source of protein for livestock feed and inland fish production. Moreover, grain legumes have the unique ability to fix nitrogen symbiotically thereby improve overall soil fertility.

Among the pulses, lentil is the most popular and ranks first in terms of human consumption in Indian subcontinent. The protein and nutritional qualities of lentil varieties have been reported to be superior to other pulse crops (Singh et al. 1968; Bhatty 1988). Thus lentil plays an integral role in agricultural systems of South Asian countries, with increasing annual demands due to its nutritional quality and popularity. Even so, this crop is typically characterized by low yield potential. Several factors are believed to be responsible for the reduced yields, including susceptibility to pests and diseases, massive flower drop, and post-harvest losses (Erskine 1984). Diseases of lentil, at various stages of growth, are caused by fungi, bacteria, viruses, and nematode infestations (Gowda and Kaul 1982).

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A number of attempts have been made to improve lentil varieties using conventional breeding techniques, but these efforts have failed to achieve desired results due to a narrow genetic base and lack of available and suitable germplasm for lentil improvement. Under these circumstances, there is considerable scope to embrace biotechnological approaches for lentil improvement. Indeed, a number of studies have been previously conducted to develop suitable *in vitro* regeneration systems, and to explore the possibility of applying biotechnology to lentil improvement programmes (Saxena and King 1987; Polanco *et al.* 1988; Warkentin and Mchughen 1993; Sarker *et al*. 2003).

However, improvement of lentil through biotechnological methods has proven to be difficult due to its recalcitrant nature, particularly pertaining to the development of an effective *in vitro* root induction system. Previous studies have indicated that rooting in microsperma type of lentil is especially difficult and appears to be a major constraint in obtaining fully regenerated plants through *in vitro* techniques (Khanam *et al*. 1995; Sarker *et al*. 2003). Micrografting has been attempted to obtain *in vitro*-derived plantlets (Gulati *et al*. 2001), but this approach was fraught with difficulties especially in case of microsperma lentil varieties. Under these circumstances, *in vitro* flowering and fully developed seed formation have been considered as a viable alternative to obtain plant progenies. It is believed that *in vitro* flowering and pod production may significantly contribute towards the genetic improvement of lentil through a biotechnological approach.

The main objective of the present investigation was to develop an alternative regeneration system for *Lens culinaris* Medik. from regenerated shoots of lentil, avoiding the *in vitro* root formation stage. The necessary first step was to determine suitable media for *in vitro* flowering and seed formation. Several reports are available on *in vitro* flowering

in a number of plant species including citrus (Moss 1969), cauliflower (Vandana *et al*. 1995), maize (Mandal *et al*. 2000), and bamboo (Nadgauda *et al*. 1990; Singh *et al*. 2000), among others, as well as *in vitro* fruit development in tomato (Sheeja and Mandal 2003). This present study is the first report demonstrating the successful development of *in vitro* flowers and viable seed formation in a microsperma lentil variety.

Materials and Methods

Plant materials and explant preparation. Two microsperma varieties of lentil (*L. culinaris* Medik.) namely, Bari Masur-1 (BM-1) and Bari Masur-4 (BM-4), typically cultivated in Bangladesh, were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, and maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka, Bangladesh.

Two types of embryo explants, namely, cotyledonary nodes and cotyledon attached decapitated embryos were used for the culture of *in vitro* shoots. For seed germination, the lentil seeds were first washed in 70 % (*v/v*) ethanol for 1 min, and then surface sterilized with 0.1 % $HgCl_2$ (*w/v*) solution for 15 min. Seeds were then rinsed three or four times with sterile distilled water. The surface-sterilized seeds were then cultured on 0.3 % (*w/v*) water-agar medium for germination. Cotyledon attached decapitated embryos were prepared from overnight soaked seeds by splitting them open and removing the root and shoot tips from each embryo. Cotyledonary node explants were prepared by removing the root and shoot tip as well as half of both cotyledons from 3-d-old germinated seeds.

In vitro shoot development. The two types of explants, the cotyledonary node and cotyledon attached decapitated embryos, were cultured on multiple shoot regeneration medium containing MS salts (Murashige and Skoog 1962) supplemented with 2.22 μM 6-benzylaminopurine (BAP), 2.32 μM kinetin (Kn), 0.29 μM gibberellic acid (GA_3), 30.35 μM tyrosine, and 3 % sucrose (*w/v*) in 250 ml Erlenmeyer flask containing 60 ml of medium (Sarker *et al*. 2003). The pH of the medium was adjusted to 5.8 with 1 M NaOH prior to adding 0.8 % agar (*w/v*), and autoclaved at 121°C for 20 min. The culture vessels were incubated in a growth room with a 16/8 h light/dark cycle at 25±2°C.

In vitro root induction. For induction of root growth, 2–4-cm-long regenerated shoots were excised and transferred to test tubes (25×150 mm) each containing 25 ml of MS or half-strength MS medium supplemented with indole-3-butyric acid (IBA) ranging from 49.2 to 123.0 μM. The test tubes were incubated in the growth room under 16/8 h light/dark cycle at 25±2°C.

In vitro flower and pod formation. To obtain *in vitro* flower and pod formation, cotyledonary nodes and cotyledon attached decapitated embryo explants were cultured for the development of shoots only. After 15 to 20 d of culture, when the regenerated shoots attained a length of 2–3 cm, individual elongated shoots were separated and cultured separately in test tubes (25×150 mm) containing 25 ml of auxin-rich MS medium for *in vitro* flower formation (Table 2). Auxins used for the induction of flowers were IBA or indole-3-acetic acid (IAA), and 1-naphthaleneacetic acid (NAA). Cultures were maintained in the growth room until flower and pod formation.

Pollen viability and observation of pollen tube growth. To examine the viability of pollen grains, *in vitro*-derived individual flowers were excised, sepals and petals were removed, and anthers were transferred to a slide and squashed in a solution containing 0.01 % (*w/v*) fluorescin diacetate (FDA) and 20 % (*w/v*) sucrose, and incubated at room temperature for 30 min (Heslop-Harrison and Heslop-Harrison 1970). Following this incubation period, debris was removed and pollen grains were observed under a fluorescence microscope.

To study the growth of pollen tubes *in situ*, the self-pollinated pistils from *in vitro*-grown flowers were collected, fixed, and softened in a mixture of acetic acid/alcohol (1:3, *v/v*) with 1 M NaOH at 60°C for 10 min, and thereafter stained with 0.1 % (*w/v*) decolorized aniline blue solution. [The decolorized aniline blue solution was prepared by dissolving 0.1 g of aniline blue in 100 ml of 0.1 M tripotassium orthophosphate (Sigma-Aldrich Chemical Co., St. Louis, MO) followed by storage of the solution in an amber glass bottle for at least 1 wk at room temperature until the solution became pale green.] After staining, pistils were mounted in one drop of 50 % (*v/v*) aqueous glycerol solution prior to microscopic examination. Stained pistils and pollen grains were observed under a fluorescence microscope fitted with an incident UV illumination system (Nikon Corporation, Tokyo, Japan, Microphot; excitation 450–490 nm) following the technique of Kho and Baer (1968).

Propagation of in vitro grown seeds. Mature seeds developed from *in vitro*-grown flowers were harvested and dried under sunlight approximately for 2–3 d. Air-dried seeds were surface disinfected and placed on water-agar medium for germination, as described previously. After 2–3 wk following germination, seedlings were transplanted into 10×6 cm plastic pots containing sterilized peat moss. The roots were gently washed free of agar using in distilled water. Pots were covered with transparent perforated polythene bags.

The inner side of these bags was moistened with water to prevent desiccation. Plants were acclimatized in the culture room at 25±2°C for 1 to 2 wk, and then further maintained in a net house for flower and pod development.

Results and Discussion

In vitro regeneration of shoots. The objective of the study was to achieve an alternative *in vitro* regeneration method for microsperma lentil varieties through the development of *in vitro* flowering and seed set. *In vitro* flowers were produced by using regenerated shoots developed from two types of embryo explants, i.e., cotyledonary node and cotyledon attached decapitated embryo, from two microsperma lentil varieties (BM-1 and BM-4). The shoot regeneration protocol utilized was based on Sarker *et al*. (2003) established for microsperma lentil varieties of BM-1, BM-2, BM-3, and BM-4. Cotyledonary node and cotyledon attached decapitated embryos produced between 3–7 and 2–5 healthy shoots per explant, respectively, on MS medium containing 2.22 μM BAP, 2.32 μM Kn, 0.29 μM GA_3, and 30.35 μM tyrosine Fig. 1*A*. Moreover, the percentage of explants with shoots was 88.7 and 74.6 in case of cotyledonary nodes and cotyledon attached decapitated embryo explants, respectively. The results obtained from the present study are consistent with the earlier report of Sarker *et al*. (2003) who observed 78.6 % shoot development from cotyledonary node explants and between 4 and 6 shoots per explant using identical microsperma lentil varieties.

In vitro regeneration techniques for lentil are not well developed compared to other grain legumes from Europe and North America, but over the last several years the situation has progressively improved. Williams and McHughen (1986) described a protocol for regeneration of lentil from hypocotyls and epicotyl-derived callus. Saxena and King (1987) obtained whole plants from callus induced from embryonic axis explants. Polanco *et al*. (1988) later reported lentil shoot formation from shoot tips in a medium supplemented with BAP and NAA. Gulati *et al*. (2001) achieved the most favorable response for multiple shoot regeneration on MS medium supplemented with 8 μM BAP and 5–8 μM GA_3. Khanam (1994) obtained *in vitro* shoot regeneration on MS medium containing 0.5 mg/l BAP, 100 mg/l CH (Casine hydrolysate), 0.5 mg/l Kn, and 0.2 mg/l NAA.

In vitro root development. Shoots regenerated from two different explants from both varieties used in this study did not produce roots spontaneously. Therefore, induction of root growth was attempted by transferring freshly regenerated individual shoots of 2–4 cm lengths to MS media

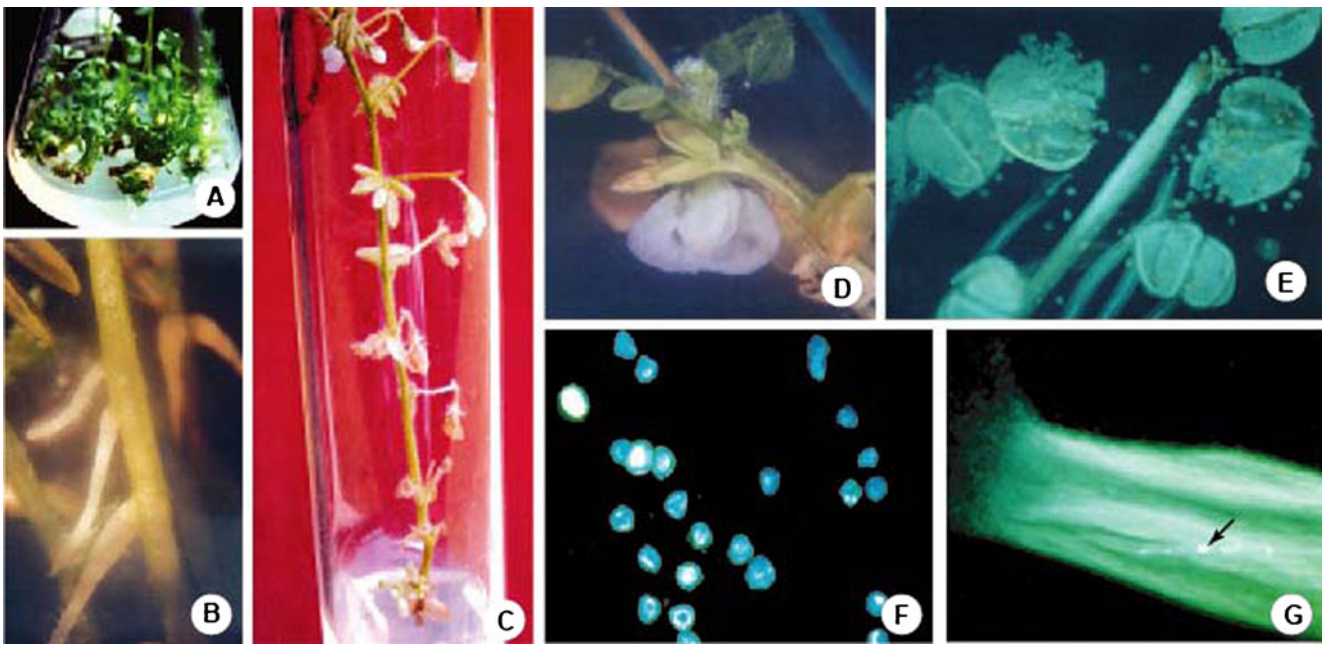

Figure 1. *In vitro* shoot formation and flower development in lentil. (*A*) Regeneration of multiple shoots from a BM-4 cotyledon attached decapitated embryo cultured on MS medium supplemented with 2.22 μM BA, 2.32 μM kn, 0.29 μM GA_3, and 30.35 μM tyrosine. (*B*) Induction of roots slightly above the cut end of the regenerated BM-4 shoots following treatment with a high concentration of IBA. (*C*) Development of several flowers from a regenerated BM-4 shoot on 1/2 MS medium supplemented with 98.4 μM IBA and 2.69 μM NAA. (*D*) A magnified view of *in vitro*-derived flower, ×8. (*E*) Anthers with large number of pollen grains, ×6. (*F*) Fluorescent microscopic views of pollen grains after FDA staining, ×10. (*G*) Callose deposits within the pollen tube (*arrow*) growing within the stylar tissue following self-pollination, ×16.

Table 1. Effect of different concentrations of IBA-supplemented MS medium on root induction in regenerated shoots of BM-1 and BM-4 lentil varieties

IBA concentration in MS media	Lentil variety	No. of shoots inoculated	% of rooting	No. of roots/shoot
49.2 µM	BM_1	22	–	–
	BM_4	30	–	–
73.8 µM	BM_1	20	–	–
	BM_4	24	–	–
98.4 µM	BM_1	30	26.7	2–5
	BM_4	35	31.4	3–5
123.0 µM	BM_1	25	36.0	3–6
	BM_4	40	37.5	3–8

supplemented with 49.2–123.0 µM IBA. Root induction was observed after 10–15 d when shoots were cultured on MS medium supplemented with 98.4 or 123.0 µM IBA (Table 1). In these cases the number of roots varied from 3–8 per shoot. However, these roots did not develop from the base of the shoots; rather they developed slightly above the excision point Fig. 1*B*, and failed to integrate effectively with the shoot vascular system. Furthermore, freshly developed roots formed callus when their tips were in contact with the medium. This result was found to be similar to that of Polanco et al. (1988) who used hormonal supplements of NAA or IAA and Warkentin and McHughen (1993) using hormone free B5 medium. Together, these reports demonstrate that rooting from *in vitro* regenerated shoots in lentil remains a serious impediment for the development of a complete *in vitro* regeneration system and successful clonal propagation of this species.

Flowering of lentil in vitro. To overcome the limitations caused by poor root development for *in vitro* shoots and the failure to obtain plantlets through these techniques, trials were conducted to initiate *in vitro* flowering and pod formation directly from *in vitro* regenerated shoots. For *in vitro* flowering, *in vitro*-derived lentil shoots were transferred to MS media supplemented with several concentrations and combinations of IAA or IBA plus NAA to induce flowering, fertilization, and seed set. For this study, at least 500 to 600 shoots were inoculated for each combination of hormonal supplements and *in vitro* flower buds initiated within 7–10 d of culture Fig. 1*C*, *D*. As presented in Table 2, the highest number (73.4 %) of flowering shoots occurred on half-strength MS medium supplemented with 98.4 µM IBA and 2.69 µM NAA Fig. 1*C*. It was observed that lower concentrations of NAA in the medium increased the number of flower buds formed per shoot, whereas increasing amount of NAA to 5.37 µM reduced the number of flower bud formation per shoot (Table 2). The number of flowers formed could be increased with two week of regular sub-culturing of the

Table 2. Effects of different concentrations of auxins on *in vitro* flowering and formation of pods with set seed

Media composition	No. of shoots inoculated	% of shoots with flowers	% of flowering shoots with pod set	Range of flowers per shoot	Range of pods per shoot
MS without auxin	570	10.52	0	0	0
MS+49.2 µM IBA	580	6.89	0	1	0
MS+49.2 µM IBA+ 5.37 µM NAA	500	60.00	25.0	1–5	1
MS+57.08 µM IAA+ 5.37 µM NAA	510	17.64	0	1–2	0
MS+49.2 µM IBA+ 2.69 µM NAA	640	68.75	22.7	1–8	1
MS+57.08 µM IAA+ 2.69 µM NAA	450	33.33	20.0	1–2	1
MS+98.4 µM IBA	405	14.81	25.0	1–3	1
MS+98.4 µM IBA+ 5.37 µM NAA	510	41.17	28.5	1–3	1
MS+114.1 µM IAA+ 5.37 µM NAA	312	9.62	0	1–2	0
MS+98.4 µM IBA+ 2.69 µM NAA	540	63.38	13.04	1–10	1–2
MS+114.16 µM IAA+ 2.69 µM NAA	525	42.85	26.66	1–4	1
1/2 MS+98.4 µM IBA +2.69 µM NAA	585	73.35	57.57	1–6	1–4
1/2 MS+114.16 µM IAA+2.69 µM NAA	525	48.57	41.17	1–4	1–3

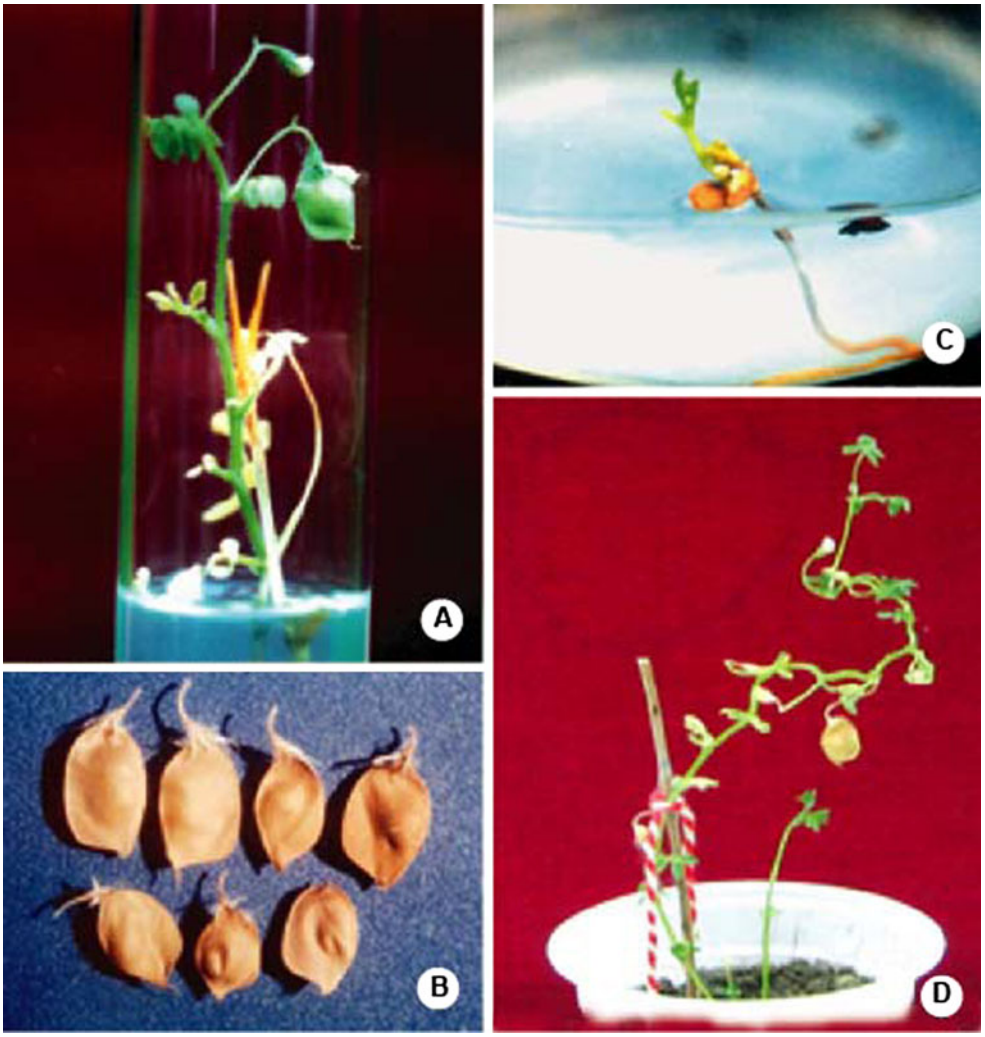

Figure 2. *In vitro* pod development. (*A*) Formation of *in vitro* pods from shoots on 1/2 MS medium containing 98.4 μM IBA plus 2.69 μM NAA. (*B*) Harvested mature pods. (*C*) Germination of *in vitro* raised seed. (*D*) Plantlet developed from *in vitro* raised seeds transferred to soil producing flowers and pods.

in vitro flower bearing shoots to fresh media having the same hormonal supplements. It was also observed that flower formation could be achieved on MS medium without any hormonal supplements, but these induced flower buds remained unopened for several weeks and failed to attain full bloom.

Anthers from *in vitro* grown flowers contained a large number of pollen grains Fig. 1*E*. The viability of the pollen grains from these *in vitro* grown flowers was tested by FDA staining. Fluorescent microscopic observation indicated that approximately 80 % of pollen grains were viable Fig. 1*F*. Self-pollinated pistils were also examined to monitor *in situ* growth of the pollen tubes within the stylar tissue. Callose deposits within healthy pollen tubes were observed within aniline blue stained pistil from the *in vitro* flowers Fig. 1*G*.

Despite the lack of reports on *in vitro* flowering and pod formation in lentil as well as in other grain legumes, a limited number of studies have been conducted in ornamental plants, as in the case of *in vitro* flowering in bitter melon (Wang *et al*. 2001), *Panax ginseng* (Tang 2000), *Murraya*

Table 3. Comparison of different explant sources for *in vitro* flowering and pod formation cultured on half-strength MS medium supplemented with 98.4 μM IBA+2.69 μM NAA

Explant type	No. of shoots inoculated	No. of shoots with flowers	% of shoots with flowers	% of shoots with pod	Days to initiate flowers	Days to pod set	Range of flowers per shoot	Range of pods per shoot
Cotyledonary node	420	340	77.00	50.00	12–15	15–20	1–8	1–4
Cotyledon attached decapitated embryo	330	210	64.66	47.62	8–10	12–15	1–6	1–5

paniculata (Jumin and Ahmad 1999), *Basilicum polystachyon* (Amutha *et al*. 2008), and *Rosa hybrida* (Kanchanapoom *et al*. 2009). Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors (Bernier 1988; Weigel 1995; Weller *et al*. 1997). On the other hand, phytohormones can also influence diverse developmental processes pertaining to *in vitro* flower formation (McCourt 1999). Previous reports indicated that *in vitro* flowering in ornamental and medicinal plants had been achieved on MS medium containing a variety of supplements including BAP and Fe^{2+} (Jumin and Ahmad 1999; Wang *et al*. 2001), GA_3 and NAA (Tang 2000), BAP and Kn (Amutha *et al*. 2008), and BAP and GA_3 (Kanchanapoom *et al*. 2009).

In vitro pod development. The flower bearing shoots were maintained by fortnightly subcultures to the same medium until *in vitro* pods had fully matured. About 12 to 20 d after flowering, pods were found to develop from healthy flowers Fig. 2*A*. Best response regarding the number of pods per shoot was obtained on half-strength MS medium supplemented with 98.4 μM IBA and 2.69 μM NAA. About 58 % flowers produced pods and the range of pods per shoot was 1–4 (Table 2).

Since half-strength MS medium successfully induced flowering and pod set, the comparative capability of the two different explants for flower development and pod formation was examined using this media only. Both varieties of lentil shoots derived from cotyledonary nodes showed a better response towards *in vitro* flower formation, without any remarkable variation between two different types of explants for *in vitro* pod formation (Table 3). It was also noted that there was no significant difference in number of flowers formed per shoot between the two varieties of lentil.

The *in vitro*-grown pods matured fully within 20–30 d under *in vitro* conditions. Pods were then harvested Fig. 2*B* and dried under sunlight. Seeds developed on *in vitro* raised shoots from both the explants of BM-1 and BM-4 were germinated on water agar-medium Fig. 2*C* with a high viability (70 %). Sixty seedlings were then transplanted into soil with 17 seedlings (28.33 %) maturing to develop flowers and pods Fig. 2*D*. The morphology of the *in vitro* raised flowers was indistinguishable from flowers grown in control fields, including the number of seeds set per pod.

The procedure of *in vitro* flower and pod formation described here can be used for the successful recovery of *in vitro* regenerated plantlets of lentil as well. Importantly it is a critical step towards the development of a transformation protocol, forming part of a lentil improvement programme.

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