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Efficient *in vitro* Direct Plant Regeneration from Mature Cotyledon Explants of Berseem (Egyptian Clover, *Trifolium alexandrinum* L.)

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ABSTRACT

Trifolium alexandrinum is a significant annual, winter, multi-cut (about 4 to 6), protein-rich forage legume crop whose genetic modification for the improvement of biomass yield and quality requires an effective *in vitro* plant regeneration protocol. In this study, a simple, efficient and reproducible regeneration system based on direct shoot organogenesis was developed using cotyledon explants. The various factors affecting *in vitro* regeneration e.g. type and concentration of cytokinin, age of donor seedling, size and explant type and genotype were optimized. Cotyledons with petiole excised from three-day old seedlings (pre-conditioned on 5 µM BAP) of *T. alexandrinum* cv. Mescavi on MSB5 basal medium with 10 µM BAP directly produced 25.88 shoots per explant in most of the cultures (94%) without the formation of intermediate callus within four weeks. The shoots were successfully rooted on NAA containing medium and the resulted plants were fertile and morphologically identical to seed raised plants. This regeneration system can be used further for transfer of agronomical important genes via *Agrobacterium tumefaciens* for genetic improvement of this crop.

Key words: *In vitro* regeneration, berseem, type and concentration of cytokinin, age and size of explants, genotype, *Trifolium alexandrinum*

INTRODUCTION

Egyptian clover or berseem (*Trifolium alexandrinum*) is one of the most important annual protein rich forage legumes grown all over the world for the cattle feed. The genus, *Trifolium* consists of approximately 237 perennial and annual plant species, of which only 25 are of agricultural significance and are commonly cultivated in broad range of habitat (tropical, sub-tropical and temperate regions) (Singh *et al.*, 2019). *T. alexandrinum* is cultivated in India, Turkey, Pakistan, Egypt and various other countries of Mediterranean origin for good quality forage (20% crude protein) with high palatability (up to 65%) and digestibility. It also helps in soil improvement by adding up to 297-400 kg/ha nitrogen to the soil. Berseem in crop rotation preserves soil as a cover crop from wind and soil erosion (Vijay *et al.*, 2017). India grows fodder crops in approximately 8 million ha area and it is hard to expound the area because of increasing need of commercial and other food crops. Using chemical fertilizers, the yield of the crop can be enhanced but it also affects the soil fertility. Biotechnology offers several techniques by which the crop yield and quality can be improved in comparison to conventional breeding techniques which are time-consuming and laborious. For quality improvement of forage crops like berseem a highly efficient and genotype independent regeneration procedure is required. Complete *T. alexandrinum* plants were regenerated *in vitro* either by direct and indirect somatic embryogenesis or organogenesis (Abogadallah and Quick 2010; Moghaieb *et al.*, 2014). Mature cotyledon explants, excised from *in vitro* raised seedlings, are available all the time without any contamination. Moreover, cotyledon explants directly regenerate into multiple shoots within a short time with no somaclonal variations. However, only few reports are

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present on regeneration of *Trifolium* species and that to at a very low regeneration frequency. This has restricted the genetic transformation of berseem (*T. alexandrinum*) by *A. tumefaciens* (Abogadallah and Quick, 2010; Moghaieb *et al.*, 2014; Anjum *et al.*, 2018). In the present study, various factors impacting the shoot regeneration in berseem using cotyledon with petiole explants have been optimized for an efficient and reproducible berseem regeneration system for its use in developing genetic transformation of Indian *Trifolium* species to improve their biomass yield and quality or to study the gene functions.

MATERIALS AND METHODS

The mature seeds of *T. alexandrinum* varieties, Mescavi, HB1 and HB2 were bought from Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana (India). Seeds were rinsed using 0.02% (v/v) Tween-20 for 5 min, surface sterilized using 70% ethanol for 60 sec followed by aqueous (0.2% w/v) $HgCl_2$ solution for 4 min. These seeds were then rinsed six times thoroughly with sterile distilled water. After blotting them dry on sterile Whatman filter paper, they were kept on medium containing full strength nutrient salts of MS - and vitamins of B5 medium [with 5 µM 6-benzyl aminopurine (BAP), sucrose 30 g/l, phytoagar 3 g/l, pH 5.8] in 90 mm Petri dishes under 16 h light and 8 h dark conditions at 25±2°C for germination. Cotyledon explants with petiole were cut from seedlings after various days of their germination *in vitro* on BAP medium (pre-conditioned) with a sterilized scalpel and forceps inside laminar air flow cabinet. The cotyledons having attached petiole were cut close to the embryonic axes and cultured with proximal end slightly embedded in medium.

MSB5 medium having different concentrations of 6-Benzylaminopurine (BAP-0, 2.5, 5, 7.5, 10, 12.5 and 15 µM) was used to optimize the effect of BAP on regeneration of shoot from cotyledon with petiole explants. Other cytokinins like thidiazuron (TDZ), kinetin and zeatin at an equimolar (10 µM) concentration of BAP were also evaluated to identify a cytokinin for inducing the maximum number of shoots.

The effect of age of explants on frequency of shoot regeneration was studied by excising the cotyledon with petiole explants from the seedlings raised on MSB5 medium with 5 µM BAP (pre-conditioned) after 16 h or 2 or 3 or 4 or 5 day of germination. These explants were cultured with cut end slightly inserted into MSB5 medium supplemented with 10 µM BAP. The cotyledon explants with or without petiole, or sliced either transversely or longitudinally, and embryonic axes without both the cotyledons were cut from the three-day-old berseem seedlings and cultured on MSB5 medium supplemented with 10 µM BAP. Hypocotyls were excised from 3-day old berseem seedlings and cultured on MSB5 medium with 10 µM BAP in vertical orientation.

Three Indian varieties of berseem. Mescavi, HB1 and HB2 were used to evaluate the effect of genotype on regeneration of shoots from cotyledon with petiole explants on MSB5 medium having 10 µM of BAP.

In all the experiments, MSB5 medium containing full strength of MS salts, B5 vitamins, 3% sucrose and 0.8% phyto-agar was used. The cultures were kept initially at 25±2°C in dark for five days and thereafter were shifted to 16 h light/8 h dark conditions of white fluorescent lights with intensity of 80 $\mu Em^{-2} s^{-1}$. The cultures were sub-cultured every forth-nightly on the same medium till the shoots developed trifoliate leaves in three weeks. The shoots were further sub cultured on MS medium carrying 0.15 mg/l BAP for 7-10 days to elongate shoots. The cultures were observed on routine basis. The regeneration frequency and the maximum number of shoots and shoot length were measured after 4-5 weeks of culture.

Fully developed 2-3 cm long shoots were detached from main explants and shifted to rooting medium i. e. MSB5 medium with 0.5 mg/l NAA for rooting. Using forceps, rooted shoots were taken out slowly from the rooting medium and washed under running tap water to remove agar completely. These rooted shoots were transplanted to earthen pots comprising soil and manure. The pots were wrapped with polythene bags for one week to maintain high humidity. Then humidity was reduced by using perforated polythene bags to reduce the humidity and plants were shifted to green house under light conditions at 25±2°C and 65% relative humidity for further growth.

Statistical analysis was performed on the average number of shoots regenerated per explant by using one-way ANOVA in Graph Pad

Prism 9.4. Data were recorded on 30 explants per treatment and each experiment was repeated three times. Tukey's 'Honest Significant Difference' test was employed for determination of significant differences between different groups at various significant levels.

RESULTS AND DISCUSSION

Cotyledon explants with petiole (Fig. 1a) excised from 3-d-old seedlings grown on MSB5 basal medium having 5 µM BAP were cultured on the same basal medium containing different concentrations of BAP i.e. 0.0, 2.5, 5.0, 7.5, 10, 12.5 and 15 µM. The explants on MSB5 basal medium did not regenerate shoot. However, addition of BAP to the basal medium led to the regeneration of multiple shoots, but the shoot length had decreased with increase of the BAP conc. (Fig. 1). The cotyledons swelled at the cut petiole end and developed shoot buds within seven days of culture (Fig. 1b). The shoot buds developed into shoots with trifoliate leaves within next three weeks (Fig. 1c, d). The cultures were sub-cultured every two weeks on same regeneration medium. The regeneration frequency as well as number of shoots increased with increase in BAP conc. up to 10 µM, thereafter, it decreased with further increase in its conc. (Fig. 2A). BAP alone at 10 µM was found to be the best for the induction of an average of 25.3±0.88 multiple shoots per cotyledon in 94% of the cultures. These findings are in corroboration with the previous reports on shoot regeneration in legumes like *Cicer arietinum, Pongamia pinnata, Arachis hypogaea, Vigna unguiculata, Vigna radiata* and *Rosa hybrida* (Tan *et al.*, 2018; Sindhu *et al.*, 2019; Aggarwal *et al.*, 2020; Sadhu *et al.*, 2020; Kumar *et al.*, 2021). In earlier reports, the maximum multiple shoot regeneration response and the number of shoots were observed from cotyledons of *T. alexandrinum* on medium with higher conc. of BAP and NAA in combination (Abogadallah and Quick 2010; Moghaieb *et al.*, 2014). Multiple shoot regeneration from cotyledons occurs from the pre-existing meristematic regions at their base on medium containing an appropriate amount of cytokinin like BAP. In the present study, a higher number of shoots with high regeneration frequency was obtained than those reported earlier. This may be due to the different cultivars used in these studies.

The morphogenetic potential of cotyledon explants excised from 16 h, 2, 3, 4 and 5-day old seedlings varied with the age of donor seedlings. The shoot regeneration frequency and number of regenerated shoots increased with increase of age of explants' donor seedlings from 16 h to 3 days, thereafter, both the parameters decreased drastically (Fig. 2B). Three-day-old explants showed the maximum, 93% regeneration frequency and produced the highest number of an average of 25±0.9 healthy shoots. Five-day-old explants regenerated only one shoot per explant. The decrease in an average number of regenerated shoots and regeneration frequency with age of the explants may be due to decrease in the reserve food in cotyledons during seed germination.

Since BAP at 10 µM induced a maximum number of multiple healthy shoots, the shoot forming response of BAP was also compared with the other cytokinins like TDZ, kinetin and zeatin at an equimolar concentration (10 µM). The shoots regenerated on MSB5 basal medium were low in number and smaller in size than those regenerated on medium containing BAP. Similarly, the length of shoots regenerated on TDZ, kinetin and zeatin containing medium could not be improved even after the prolonged culture. But these cytokinins exhibited poor shoot forming response compared to BAP. TDZ was second to BAP in inducing more shoots followed by kinetin (4 shoots per explant) and zeatin (3 shoots per explant). BAP was the most effective shoot forming cytokinin as reported earlier by other (Abogadallah and Quick 2010; Moghaieb *et al.*, 2014; Anjum *et al.,* 2018). The shoot forming response of BAP was not improved when it was used in combination with auxin (NAA) at different concentrations. Similar result has been reported in other legumes like *Vigna unguiculata* (L.). Walp. where 2.5 mg/l BAP induced maximum number of shoots (Chekroun and Belkhodga, 2017). Thus, BAP was most effective in developing multiple shoots from explants (Fig. 2C).

The regeneration capacity of plants varied with the genotype. It was, therefore, necessary to identify a most regenerative variety for its use in genome manipulation and gene editing. Three varieties of *T. alexandrinum,* Mescavi,

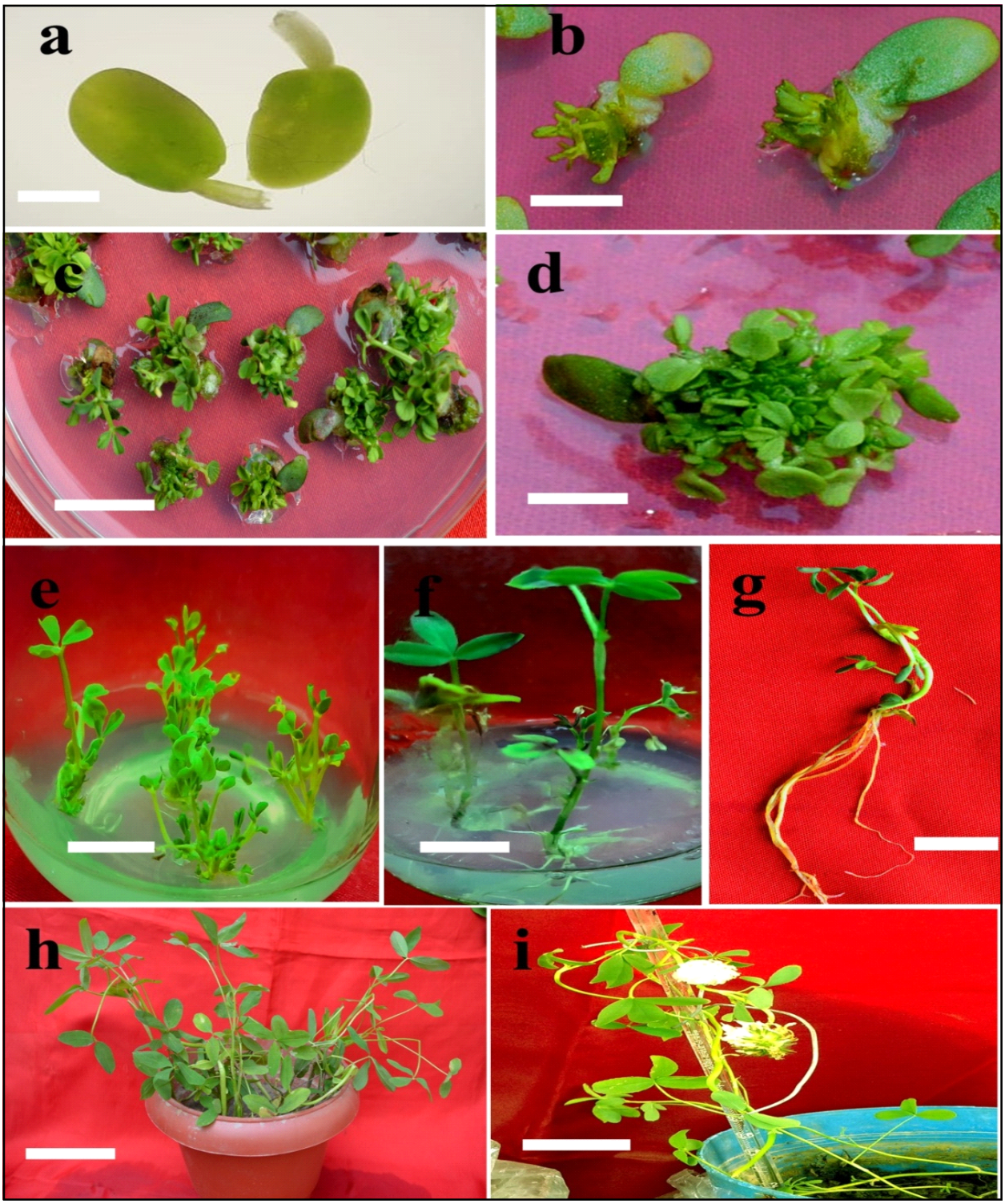

Fig. 1. *In vitro* shoot regeneration from cotyledon explants with petiole of *Trifolium alexandrinum*: (a) cotyledon explants with petiole excised from 3-d-old seedling germinated on BAP medium (scale bar = 0.5 cm); (b, c) direct regeneration of shoots from the cut petiolar end on BAP containing medium after 12 days (scale bar = 0.5 cm); (d) regeneration of multiple shoots on MSB5+10 µM BAP medium after two weeks; (e) shoots were sub-cultured on shoots elongation medium (MSB5+0.15 mg/l BAP) (scale bar = 1 cm); (f) elongated shoots on rooting medium, MSB medium containing 0.5 mg/l NAA (scale bar = 1.5 cm); (g) a plantlet with fully developed roots (scale bar = 1 cm); (h) plantlets established in soil (scale bar = 5 cm) and (i) well-established plants with white coloured flowers (scale bar = 1 cm).

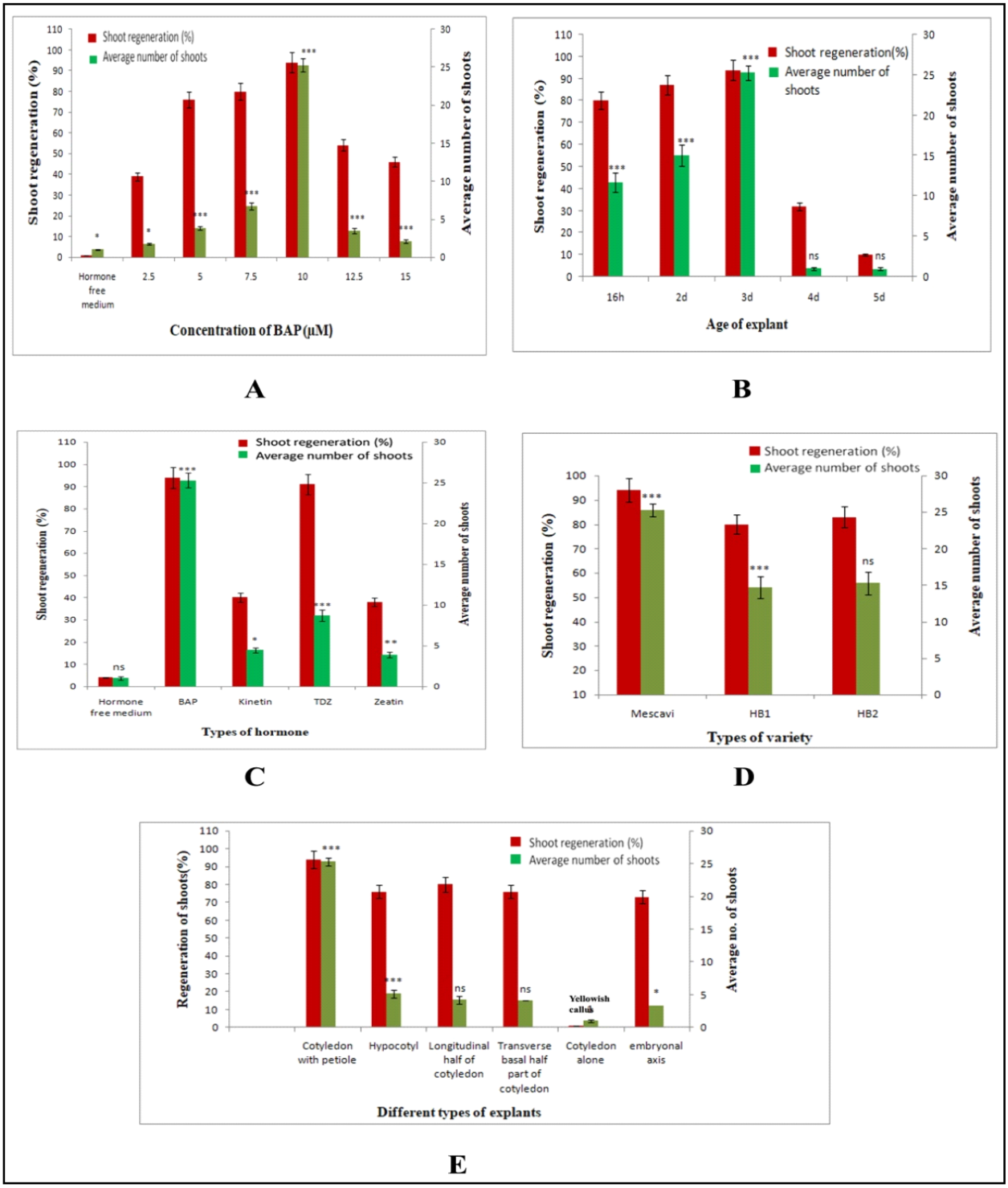

Fig. 2. Optimization of *in vitro* shoot regeneration system of *Trifolium alexandrinum:* (A) Effect of different concentrations of BAP on % regenerated shoots and average no. of shoots from 3-d-old cotyledon explants with petiole of *Trifolium alexandrinum* variety Mescavi; (B) Effect of age of explants on *in vitro* % regenerated shoots and average no. of shoots regenerated per explant; (C) Effect of equimolar (10 µM) concentrations of different cytokinins on *in vitro* regeneration of shoots (%) and an average no. of regenerated shoots per explant; (D) Effect of different genotype (variety) on an average no. of shoots per explant and (%) shoot regeneration and (E) Effect of different types of explant on shoot regeneration and an average no. of shoots produced per explant. Significant differences were obtained at various significant levels *P<0.05, **P<0.01 and ***P<0.001 using Tukey's HSD test.

HB1 and HB2 were used in the present study to determine the most regenerative one (Fig. 2D). Cotyledon explants with petiole from 3-day old seedlings of three varieties were regenerated on MSB5+BAP (10 µM) medium. Mescavi was found to be the most responsive for producing the maximum regeneration frequency (94%) with a maximum of 25.8 shoots followed by HB2 and HB1 with 83 and 80% regeneration frequency and 15.3 and 14.7 shoots per explant, respectively.

Different explant types possess different regeneration potential. In the present study, different explant types, entire cotyledons with or without petiole, longitudinally or horizontally sliced halves of cotyledons were regenerated on MSB5 basal medium with 10 µM of BAP (Fig. 2E). Cotyledon explants with petiole were found to be the most regenerative and produced a maximum number of shoots (25.8) with a high regeneration frequency (94%). Cotyledons without petiole did not produce any shoots. Both the transverse (proximal) or longitudinal halves of cotyledon explants produced 4-5 shoots with 73% regeneration frequency.

Hypocotyl explants excised from 3-d-old seedlings and cultured in vertical position on MSB5 medium with 5 µM BAP plus 2 mg/l NAA. The proximal (close to cotyledonary node) end of hypocotyls directly produced 5-6 multiple shoots, while distal end formed only callus. Hypocotyls used for indirect shoot regeneration showed lower shoot induction frequency (8%) in comparison to petiole (more than 12%) of *T. alexandrinum* (Moghaieb *et al.*, 2014). Embryonic axes explants excised from germinated 16 h water soaked seeds on regeneration medium, MSB5 with 5 µM BAP, produced 4-5 shoots in each explant within 21 days.

Regenerated shoots (3-4 cm in length) on root induction medium (MSB5 medium having 0.5 mg/l NAA) initiated healthy roots within 10-12 days (Fig.1f) which were subsequently elongated considerably in the next two weeks time (Fig.1g). However, NAA at a higher concentration (1-2 mg/l) was used for rooting in earlier reports (Abogadallah and Quick 2010; Moghaieb *et al.*, 2014). The plantlets established in pot containing soil resumed growth and produced flowers and seeds. The tissue cultured raised plants were similar in morphology to the seed derived plant (Fig. 1h, i).

CONCLUSION

A simple, efficient and reproducible regeneration protocol for berseem *T. alexandrinum* cv. Mescavi using cotyledon explants was developed. Cotyledons with petiole excised from 3-day old seedlings (pre-conditioned on 5 µM BAP) on culture on MSB5 basal medium with 10 µM BAP directly produced 25.88 shoots per explant in maximum cultures (94%) without the formation of intermediate callus within four weeks. The shoots were successfully rooted on NAA containing medium and the resulted plants were fertile and morphologically similar with seed grown plants. The developed protocol can be used for genome engineering and editing for the qualitative and quantitative improvement of berseem.

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