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EFFICIENT *IN VITRO* PLANTLET REGENERATION FROM COTYLEDON EXPLANTS IN SUGAR BEET (*BETA VULGARIS* L.)

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ABSTRACT

An efficient *in vitro* regeneration technique was established in sugar beet (*Beta vulgaris* L.) using cotyledon as explants from *in vitro* raised seedlings. Regarding plant growth regulators added in inoculation media showed that BA in combinations with NAA was most responsive for multiple shooting in which, 90% of shoot emergence from inoculated explants were observed with a medium containing 0.5mg/L BA+0.5mg/L NAA at 30 days of inoculation period. The highest number of shoots per explant was 7.5± 0.56 and the maximum shoot length of 6.5±0.25cmwere observed in this medium inoculation after 90 days. *In vitro* raised shoots rooted well when they were transferred to MS + 3.0mg/LIBA in which, 70% shoots were rooted in this medium transferring after 30 days. The maximum number of roots per shoot was 5.50±0.46 and the average root length of 5.8 ±0.62 cm were recorded inoculation after 45 days. About6 months were needed from culture initiation to plantlets establishment in natural environment and 55% plantlets were found to be acclimatized and resumed a new growth.

Keywords: Sugar beet, Cotyledon, Regeneration

INTRODUCTION

Plant tissue culture techniques have become a powerful tool for sustainable production of plant-derived additives with application in food and cosmetic products (Krasteva *et al*., 2021). In the past decades, micropropagation and other *in vitro* techniques have become more widely used in commercial horticulture and agriculture for mass propagation of crop plants (Twaij *et al*., 2020;

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Espinosa-Leal *et al*., 2018; Das *et al*., 1996). Sugar beet is the major sucrose producing crop grown intemperate region whilst sugarcane is an important agricultural cash crop in tropical and subtropical region of the world including Bangladesh. Sugarbeet contributes approximately 37% sucrose (Bekheet *et al*., 2007)with a high yield potential of 24 t sugar ha^{-1} (Hoffmann and Kenter, 2018) and the rest more than 60% comes from sugarcane for world's sugar production (Guimarcesand Sobral, 1998). Nevertheless, there is immense potential of adapting sugar beet in subtropical region like Bangladesh. Due to biennial and allogamous nature of the crop, it is needed to conserve and multiply the superior genotypes for breeding and genetic studies. Sugar beet is mainly propagated by seeds and root cuttings. These techniques are not suitable for tropical and subtropical regions as it does not produce seeds in this climacteric condition.

Thus, tissue culture technique could be an alternative and effective method for large scale and rapid multiplication of an elite clone for safe cultivation of this crop (Chokheli *et al*., 2020; Roy *et al*., 2017; Sarkar *et al*., 2016). In Bangladesh, sugar beet is not cultivated commercially due to lake of propagules and complex method of sucrose extraction. Despite, limited cultivation is practiced for the selection of heat tolerant line and creation of new variety through biotechnological approaches. Therefore, this study will discuss the regeneration capacity of *in vitro* cotyledon culture of sugar beet which will facilitate the future research to develop sugar beet variety for cultivation in the country. The development of an *in vitro* regeneration protocol of sugar beet from different explants tissue with genetic improvement and molecular analysis have been reported by several authors such as from leafaxil and petiole (Ritchie *et al*.,1989, Tetu *et al*.,1987), from leaf tissue (Ferytag *et al*.,1988), from cotyledon and leaf tissue (Van and Jacobs, 1985), from mesophyll tissue (Bhat *et al*.,1986), from axillary buds (Mezei *et al*.,2006; Mezei *et al*.,1990, Mezei and Kovacev, 1991), from apical meristem (Goska and Szota, 1992), from inflorescence pieces (Zhong *et al*.,1993), from shoot tips (Bekheet *et al*., 2007), from flower bud and auxiliary bud (Mikami *et al*.,1985), from petiole explants (Gurel*et al*.,2003) and from hypocotyls and cotyledon explants (Gorel *et al*., 2000).

MATERIALS AND METHODS

Plant materials

For the establishment of *in vitro* regeneration protocol of sugar beet *(Beta vulgaris* L.), freshly matured and dried seeds were collected from the local market. Seeds were washed thoroughly under running tap water to eliminate dust and surface contaminants. Then the seeds were washed with sterile deionised water for 3-4 minutes followed by washing in 70% ethanol for 30 seconds. Seed were surface sterilized with 0.1% $HgCl_2$solution for 10 minutes and thoroughly washed three times with sterile deionised water.

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Growth conditions

Surface sterilized seeds were plated on full strength Murashige and Skoog (MS) medium and transferred into a growth chamber with 16 h light and 8 h dark, 24°C temperature, 70% relative humidity and photosynthetic active radiation 1300 μE m^{-2} s^{-1} for germination.

Explant preparation

Cotyledons were aseptically excised from 15-day-old *in vitro* grown seedlings and used as explants for micro propagation. Cotyledons were separated from axenic seedlings and inoculated on MS medium containing different concentrations and combinations of plant growth regulatorsincludingindole-3-butyric acid (IBA), kinetin and 1-naphthylacetic acid (NAA) for multiple shoot formation. Subcultures were done on each respective medium at 21 days interval for promoting strong and healthy multiple shoots. Healthy shoots were then excised individually and transferred to MS media supplemented with different concentrations of IBA, NAA and IAA for root induction. The media contained 3%sucrose, 0.7%Difcobacto agar and the pH of the media adjusted to 5.8 prior to auto claving. Cultures were incubated at 26°C with 70-80% relative humidity in16 h illumination of 10,000 lux provide by cool white fluorescent light. The experiments were conducted with 10 explants per media type and replicated thrice. Data were collected on different characters at day 90 for multiple shooting and at day 45 for rooting of *in vitro* raised shoots. Observations on cultures were carried out daily.

Statistical analysis

The experiments were arranged in a completely randomized design (CRD). A descriptive analysis was carried out using the recorded data. Each value represents mean ±standard errors.

RESULTS AND DISCUSSION

In this study, different concentrations and combinations of plant growth regulators were added to culture media and investigated for direct multiple shoot induction of sugar beet. Results revealed that the highest percentage (90%) of multiple shoot formation from explants was found on medium containing MS + 0.5 mg/L BA + 0.5mg/L NAA on 30 days after inoculation (Figure 7a).In this medium, the explants produced the highest (7.5 ± 0.56) number of shoots with the maximum (6.5 ± 0.25 cm) shoot length on 90 days after inoculation (Figure 7b, c). Plant growth regulators added to the culture media, cytokinin (BA) in combinations with auxin (NAA) were found more effective for direct multiple shoot induction in cotyledon explants of sugar beet. Similar observations were reported by Bekheet *et al*., 2007 using shoot base as explants, Mezei and Kovacev,1991 using cotyledon and leaf tissue as explants and Gurel *et al*., 2003 using petiole as explants. The obtained results in the above-mentioned studies are inconsistent,

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probably due to the different explants used or the effect of genotypes or different hormonal concentrations and combinations used. On the contrary, Mishutkina and Gaponenko (2006) reported promising results using growth regulators of Zeatin, Kinetin and BA separately indifferent explants and genotypes which supports the present investigation. The combined actions of cytokinin and auxin gave rise to best multiple shoot formation (Figure 7). Direct shoot regeneration in sugar beet were also reposted previously from various explants (Freytag *et al*., 1988, Krens and Jamar, 1989, Miedema, 1982 and Rady, 1998). In this study, addition of BA single and in combinations with BA + Kinetin with the shoot multiplication media showed less responsive and in some of this media combinations explants did not response to produce any multiple shoots. This might be due to the imbalance media composition used that leads toxic to the explant tissue. Malformation of shoots also observed with the prolong (6 to 12 months) subcultures of explants on 0.5mg/L to 1.5mg/LBA in MS supplemented media. As we know that auxins play an important role for adventitious root formation of *in vitro* raised shoots of many plant species. Different plant species may vary in their requirement of auxin type and their concentrations for adventitious root formation.

Among the auxin used in this study, IBA was found to be the most responsive for root induction of *in vitro* raised shoots of sugar beet (Figure 2). Among the IBA concentrations tested, it was found that 3.0 mg/L was optimum, in which 80% of the shoot induced roots (Figure 8a). The number of roots per shoot was 5.5 ± 0.42 and the average root length of 5.8 ± 0.62 cm were observed in this medium (Figure 8b, c). It was also observed that increasing of auxin concentrations on media showed promotion of root induction. This indicates that relatively higher concentration of auxin (IBA) is suitable for root development of sugar beet *in vitro*. The present investigation is in accordance with the studies reported by Bekheet *et al*., 2007, Goska and Rogozinska, 1990 and Mikami *et al*., 1985. On the other hand, Mezei *et al*., 1990 obtained best *in vitro* rooting of sugar beet on auxin free medium where as Gurel *et al*., 2003 and Gurel and Wren 1995 found NAA more effective for *in vitro* root formation of sugar beet. These might be because of different genotypes or explants used in their studies. The superiority of IBA for *in vitro* rooting over other auxins has also been reported (Jaiswal and Amin, 1987, Amin *et al*., 1992, Amin and Akhter, 1993; Grewal *et al*., 1994; Zhang et al. 2017). Comparatively healthy rooted shoots were taken out from the culture vessels and washed gently under running tap water to get rid of agar. The *in vitro* rooted plantlets were then transferred to earthen pot and poly bags containing a mixture of soil and compost (2:1) and covered with transparent polythene to maintain humidity. Polythene cover was removed from the plantlets after one week. The plantlets were kept in a shade and misted twice a day to adapt at the natural environment (Figures 1-6). About 70% plantlets were survived in outdoor condition. The protocol for micropropagation method was developed in this study is useful and applicable to other genotypes of sugar beet. It also helps to provide propagules for cultivating of this crop in homestead area.

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Figures 1.6: *In vitro* shoot initiation and plant regeneration from cotyledon explants of sugar beet. (1) Multiple shoot initiation on MS + 0.5 mg/L BA + 0.5 mg/L NAA after 1 month of culture medium, (2, 3) shoot elongation and healthy shoot formation in the same medium after 3 months of culture, (4) root induction on MS + 3.0 mg/L IBA after 1 month of culture medium,(5)*in vitro* raised plant resumed new growth in the earthen pot,(6) *in vitro* raised plant growing in the field.

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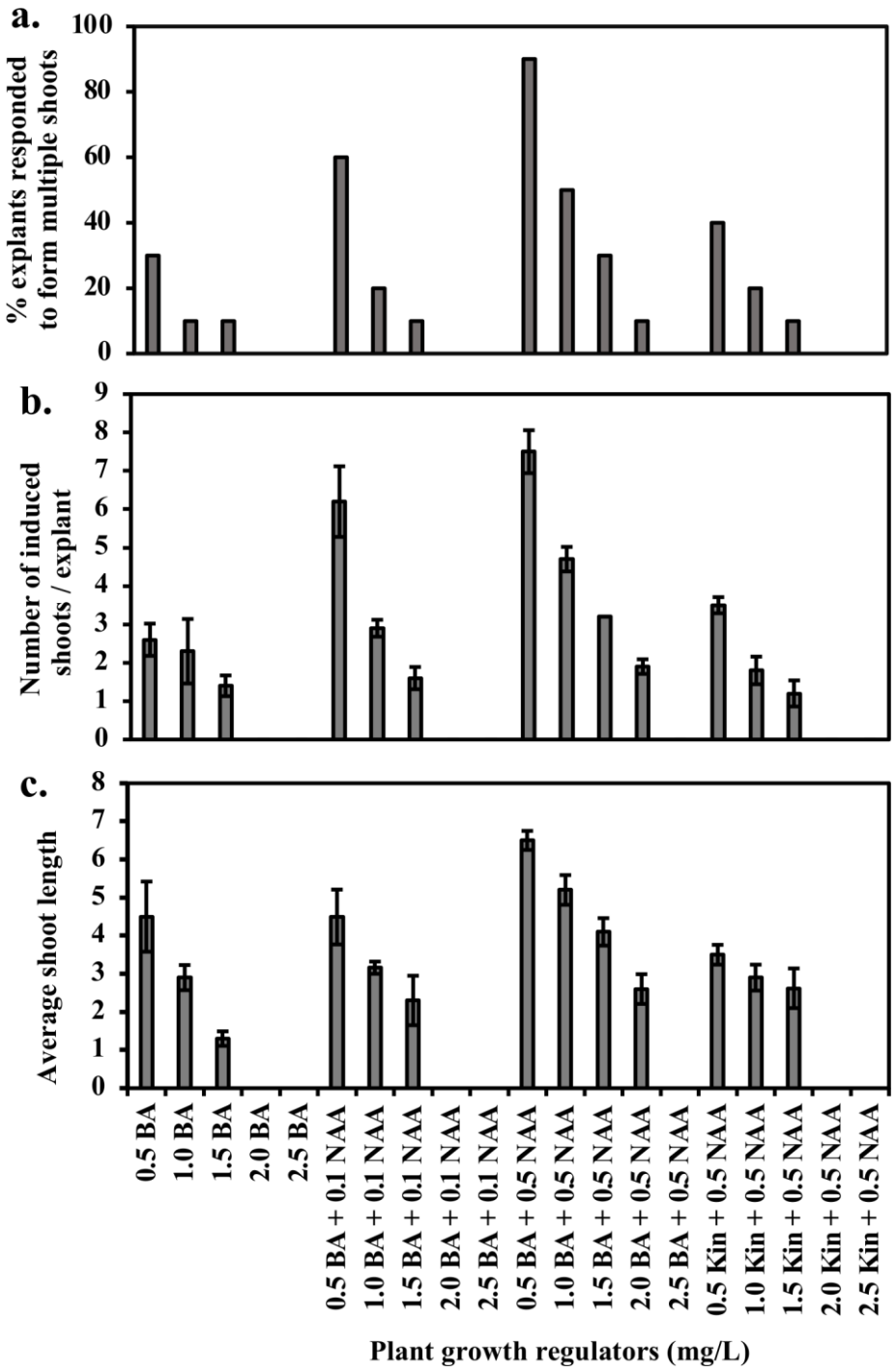

Figure 7: Effect of different concentrations and combinations of plant growth regulators on MS supplemented media for *in vitro* multiple shoot induction of sugar beet inoculation after 90 days.

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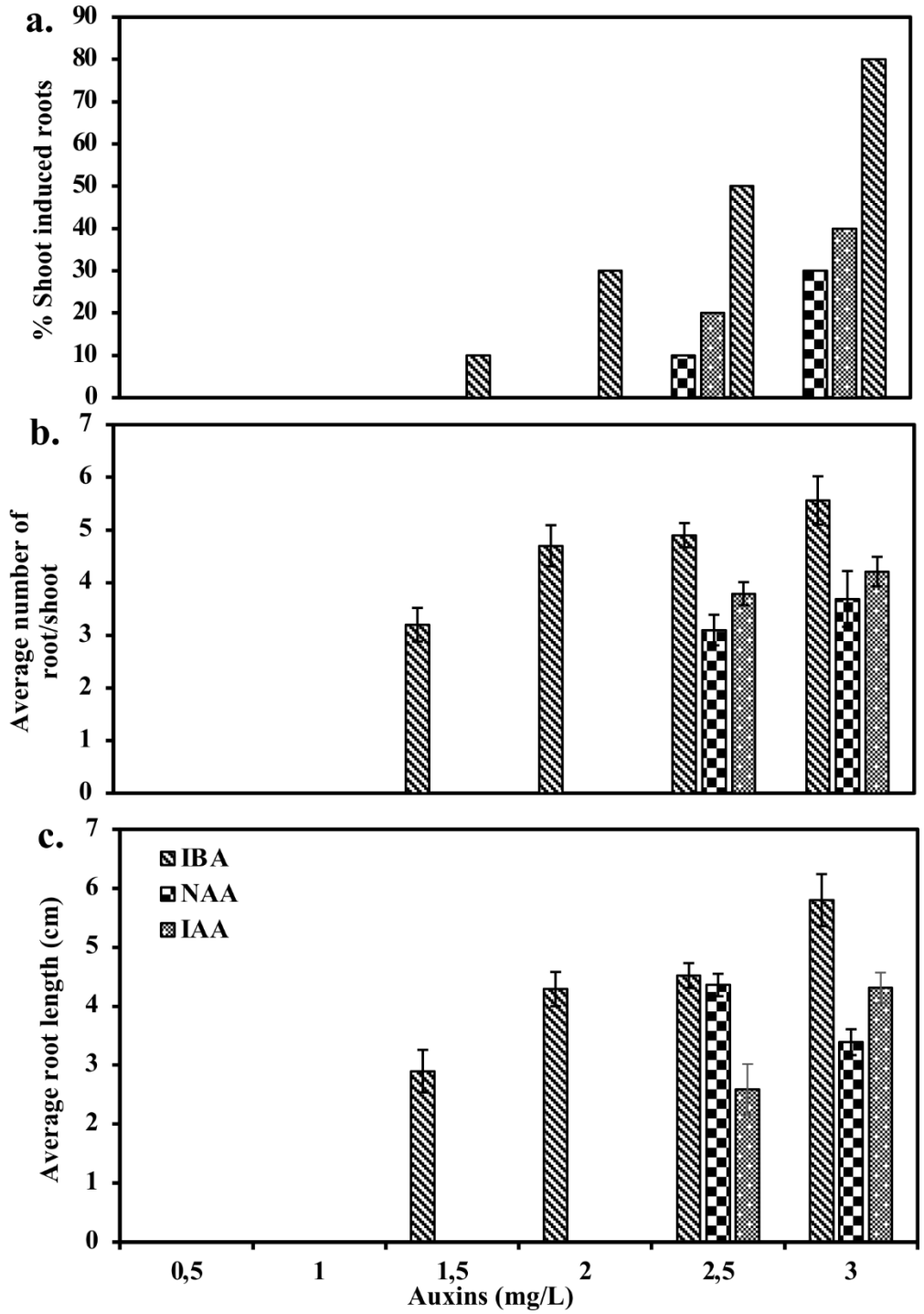

Figure 8: Effect of different auxins and their concentrations for root induction of *in vitro* raised shoots of sugar beet inoculation after 30 days.

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