ISSN 2250-3153

Effect of plant growth regulators on micro-propagation of Sunflower (*Helianthus annuus L.*)

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Abstract- Three different explants; leaf, root and stem were taken from *in vitro* grown seedlings to observe shoot regeneration, callus induction and rooting Sunflower (*Helianthus annuus* L.). Shoots were regenerated on MS basal medium containing 0.1mg/l α-naphthalene acetic acid (NAA) with six different concentration of 6-benzylaminopurine (BAP) (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0mg/l). The regenerated shoots were induced root on MS basal medium with Indole-3- butyric acid (IBA) (0.01, 0.1, 1.0 mg/l). Callus was induced on MS basal medium with 0.1 mg/l BAP + 2, 4 D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0mg/l). Completely Randomized Design (CRD) with 10 replicates was used. Data was analyzed using SAS program (9.1.3**).**

After 30 days the numbers of regenerated shoots, callus diameter, time for callus initiation and shoot regeneration, number of roots and mean root length were evaluated. Observations indicated that there were a significant treatment effects at $p<0.05$ level. Significantly highest number of shoots (2.5 shoots/explant) was observed from stem explant in MS basal medium with 0.5 mg/l BAP + 0.1mg/l NAA. Shoots were highly responded for root induction at 1.0 mg/l IBA. Significantly highest diameter of callus (1.79 cm) was observed at 2.0mg/l 2, 4-dichlorophenoxy acetic acid (2, 4 D) with 0.1 mg/l BAP. Result of this study revealed, among three types of explants, the highest response for shoot regeneration and callus production observed from stem explants in *Helianthus annuus* L.

Index Terms- *Helianthus annuus* L, regeneration, callus induction, root formation

I. INTRODUCTION

Sunflower (*Helianthus annuus* L. - Asteraceae) has heliotropic movement of the sunflower's head and it is now fairly widely cultivated throughout the world (Cavallini *et al*, 1997). Sunflower is cultivated mainly as a staple crop for edible oil production as well as a source of protein and bio renewable energy. However this species is used as an ornamental plant due to attractiveness flower (Mayor *et al*, 2010). Sunflower oil has high linoleic acid content. Linoleic acid is required for the cell membrane structure, cholesterol transportation in the blood and for prolonged blood clotting. Hence, Sunflower oil helps to reduce the serum cholesterol levels.

Sunflower is an annual herbaceous crop that is propagated by seed only. The cultivars grown were open-pollinated and cross-pollinated mostly by insects. Self-incompatibility can be seen in sunflowers which is why they rely heavily on pollen movement between plants by insects and bee colonies. Poor seed germination is one of major problem in sunflower cultivation and also stratification is required to induce seed germination. To attain sustainable sunflower productions, some constraints have been addressed by conventional breeding and enhanced management but it has resulted in limited commercial success. The integration of modern biotechnology like plant tissue culture into breeding programs may provide powerful tools to overcome these limitations.

The application of biotechnological methods for improving the characteristics of sunflower is limited mainly by the difficulty of regenerating plants in a reproducible and' efficient way. Sunflower organogenesis is highly variable and depends upon genotype, specific media components and the nature of the explant. Over the last few years, a variety of techniques for regeneration by organogenesis [Berrios, E.F (1999), Chraibi, K.M.B (1992), Witrzens, B.T (1988), Mohammad Sharrif Moghaddasi, (2011)] or somatic embryogenesis [Mohammad Sharrif Moghaddasi (2011), Nestares, G., R (1996)] have been described in this species. Plant regeneration parameters have been shown to be under quantitative genetic control in sunflower [Berrios, E.F (1999), Sarrafi, A.R (1996)]. Sunflower tissue culture has been doing since two or three decades. The first report of successful plant regeneration from sunflower callus was observed by Sadhu, (1974). By growing stem pith on a modified White's medium with 1 mg/l IAA, callus was induced. After 10 weeks, one piece of callus differentiated into several plantlets. Greco *et al*., (1984) showed that callus capable of regeneration can be obtained from every part of the seedlings (except roots). Paterson & Everett (1985) reported embryogenesis of sunflower inbred from 12 day old seedling hypocotyl explants on a modified MS medium (supplemented with 6.9 g/l total KN03, 40 mg/l Adenine sulphate, 500 mg/l casamino acids, 1.0 mg/l BA, 1.0 mg/l NAA and 0.1 mg/l GA3). Cavallini & Lupi (1987) studied cytological condition of *in vitro* shoot apical meristem derived calli and regenerated shoots. Knittel, *et al*., (1991) obtained high frequency plant regeneration from mature sunflower cotyledons. Pugliesi, *et al*, (1991) showed plant regeneration and genetic variability in tissue cultures of sunflower cotyledons.

Present study was conducted to select most suitable explants source for rapid shoot multiplication, appropriate hormone concentration and their combinations for proper shoot proliferation, callus induction and induce rooting. The develop protocol an "efficient *in vitro* propagation for *Helianthus annuus* L" will be beneficial for future plant breeding aspects.

II. METHODOLOGY

Sunflower seeds (variety 345) were purchased from Onesh Agri (Pvt) Ltd. Kent Road, Colombo. Seeds of *Helianthus annuus* L were washed with soap water and running water for 1/2 – 1 hr. Then surface sterilized by immersion in 70% ethanol for two minutes. After that seeds were surface sterilized using 20% clorex for 20 minutes in laminar air flow cabinet. Surface sterilized seeds were rinsed 3 times with sterile distilled water and they were dried onto sterile filter papers. Seed coat was removed using sterile scalpers and pliers. Seeds were introduced to glass tubes (1 seed/tube) containing 5ml of hormone free Murashige and Skoog's basal medium (MS). Three weeks old *in vitro* seedlings (10cm height) were used to take explants.

After 14 days, stem, leaves and root were dissected from *in vitro* grown seedling as explants. Stem and roots cut into 0.5 cm long pieces and leaves were cut transversally into two parts and then cultured on Murashige and Skoog (MS) medium supplemented with 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3mg/L BAP with 0.1mg/L NAA for shoot induction. (4 explants/ Jam bottle containing 20ml MS medium). Explants were cultured on MS medium supplemented with 0, 0.5, 1.0, 1.5, 2, 2.5, 3mg/L 2, 4 D with 0.1mg/L BAP for callus induction. (1 explants/glass tube containing 4ml MS medium). After that developed callus were transferred to the MS basal medium with different BAP concentrations to regenerate. The type of explants highly responded for the shoot and callus induction was observed. Regenerated shoot were rooted in MS basal medium supplemented with different concentration of IBA (0.01mg/l, 0.1mg/l and 1.0mg/l).

All medium solidified by 0.8% agar and the p^H was adjusted to 5.8- 6.0 using 0.1M NaOH. All media were autoclaved at 121^0C for 20 min. The cultures were maintained at a temperature of 24^0C ± 1^0C under continuous fluorescent light ($35\mu Em^{-2}s^{-1}$). Treatments were replicated 10 times and number of shoots per explants and time taken to regenerate, diameter of callus and time taken to initiate callus (days) and number of roots per shoot were recorded.

III. STATISTICAL ANALYSIS

The experiment was arranged in a Completely Randomized Design (CRD). Data was analyzed using SAS program (9.1.3**).** Data were subjected to analyses of variance and the mean comparison was done using Duncan Multiple Range Test (DMRT) at 5% significance level.

IV. RESULT AND DISCUSSION

Effects of plant growth regulators on shoot induction

Shoot initiation could be observed in stem explants while both leaves and root explants not responded for shoot regeneration. The highest mean number of shoots per stem explants (2.5) produced 0.5mg /l BAP with 0.1mg /l NAA (fig. 2). There was no significant difference in shoot production between the treatments with BAP at 1.0, 1.5mg/l with NAA 0.1mg/l. However no any shoot production observed in BAP at 0.0, 2.0, 2.5, 3.0 mg/l with NAA 0.1mg/l. The stem explants of *Helianthus annuus* initiated shoots during the shortest time (7 days) at 0.5mg/L BAP and 0.1mg /l NAA (fig 1 and fig. 5b).

Similar results were observed from Hayati Arda (2004), different Hybrid of *Helianthus annuus* L was given the best result at the 0.5mg/l BAP and 0.1mg/l NAA by culturing cotyledon leaves, hypocotyls, leaf and root explants for the shoot generation. According to Ekrem *et al* (1998), Shoot tip explants of cv. Hysun 45 was given best result for shoot initiation at the 0.5mg/l BAP with 0.1mg/l NAA. But shoot tip explants gets maximum number of shoots in the 2.0mg/l BAP without NAA. According to Ozyigit *et al*, (2005, 2007) recorded that the best condition for shoot induction of sunflower was found to occur on MS medium with 0.5mg/l NAA combined with 1mg/l BA. But in the current study, best result observed in MS basal medium with 0.5mg/l BAP and 0.1mg/l NAA.

Auxins are particularly included in a culture medium to stimulate callus production and cell growth, to initiate shoots, particularly roots and to induce somatic embryogenesis. Cytokinines (BAP) have been reported to induce the development of axillary buds and adventitious buds through decreasing apical dominance (Taji *et al*, 1995). When cytokinin was higher than the auxin level, shoot development was promoted. Similar results were observed in the present experiment. Presence of cytokinin at a relatively high level with low level of auxin promoted the shoot multiplication.

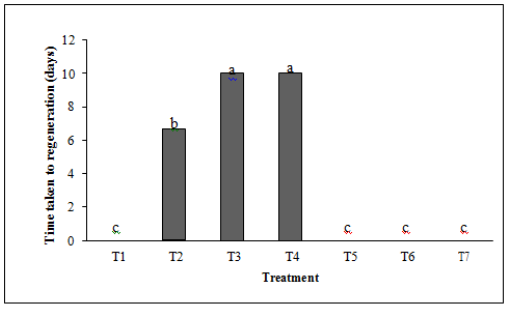

Fig. 1 Time taken to regeneration shoot on different hormone combination of BAP (mg/l) and NAA (mg/l). Mean with the same letters are not significantly different at P<0.05

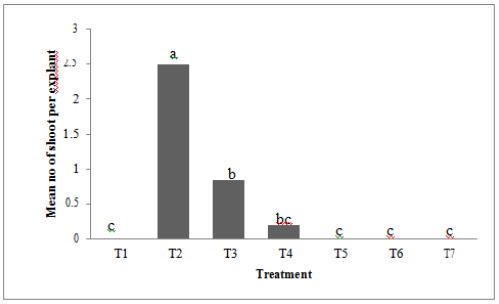

Fig. 2 Effect of different hormone combination of BAP (mg/l) and NAA (mg/l) on shoot regeneration of *Helianthus annuus* L using stem explants. Mean with the same letters are not significantly different at P<0.05

Effects of plant growth regulators on callus induction

Callus induced at 2, 4-D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) with 0.1 mg/l BAP from stem and leaf explants. Root was not responded for callus formation in any tested hormone

concentration. In both stem and leaf explants, callus initiation was significantly ($p<0.05$) high in MS basal medium containing 2mg/l 2, 4 D and 0.1mg/l BAP. But callus inductions from stem were faster than leaf. Callus induction of stem get shorter time (7.2 days) compare to leaf explants (12.9 days) (fig. 3, fig.4 and fig. 5c,d).

Similar results were observed from Pandurang *et al* (2012), callus was inducted from various explants viz., cotyledon, hypocotyls and leaf explants of *in vitro* grown plantlets in sunflower. 2, 4-D and NAA were used for callus induction in the range of 1-3 at 2 and 3mg/1 responded well for callus induction and growth NAA did not favored much callus induction at any of the concentration used. According to Ekrem *et al* (1998), shoot-tips, hypocotyls, cotyledons and cotyledonary petioles were enhanced the callus induction in the combination of 2.0 mg/l 2, 4 D and 0.1 mg/l BAP. According to Maria Carla Lupi, (1987) Hypocotyls and cotyledon explants formed callus on medium containing 2mg/l NAA and 0.5mg/l BA. Ozyigit *et al* (2005, 2007) observed callus can be obtained from different explants with various plant hormones and their combinations. For callus induction, MS + 1mg/l 2, 4 D were used. But in the present study, highest callus was induction was observed in 2 mg/l 2, 4 D and 0.1mg/l BAP.

Result of this study revealed, among three types of explants stem, leaf and root highest response for shoot production observed from stem explants in *Helianthus annuus* L. Callus induction from stem explants was faster than leaf and root. In sunflower tissue cultures, hypocotyl and cotyledon explants are good regeneration materials that show different regenerative behavior when kept in a culture, depending on their genotype (Ozyigit *et al*, 2007). The similar results were observed in our study; shoot initiating visually observed in stem explants. Both leaves and root explants not responded for shoot regeneration in all tested hormone combinations. Callus induction from stem explants was faster than leaf and root.

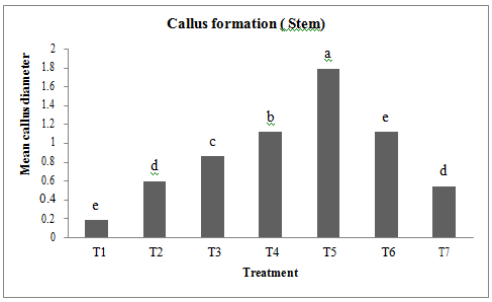

Fig. 3. Effect of different hormone combination of 2, 4 D (mg/l) and BAP (mg/l) on callus induction of *Helianthus annuus* L using stem explants. Mean with the same letters are not significantly different at P<0.05

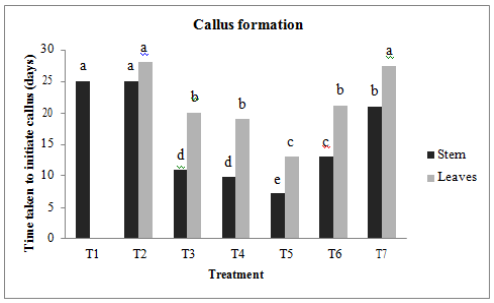

Fig. 4 Time taken to initiate callus in different hormone combination of 2, 4 D (mg/l) and BAP (mg/l). Mean with the same letters are not significantly different at P<0.05

Effect of plant growth regulators on root induction

MS basal medium with IBA significantly ($p<0.05$) affected to develop roots 0f *Helianthus annuus* L. The highest mean number of roots per explants (6) and mean root length (3 cm) was observed in 1.0mg/l IBA. But of 0.1mg/l and 1.0mg/l IBA not significantly ($p< 0.05$) affected for the root length (Fig 5e and fig. 6). The lowest mean number of roots per explants (1) and mean root length (6 cm) was observed in 0.01mg/l IBA. *Helianthus annuus* had highest root induction at 1mg/l IBA. According to Ozyigit *et al*, 2007 roots were thicker and denser on MS medium supplemented with 1mg/l IBA than hormone free MS medium. Similar results were observed in present study too, thicker and dense root induction could be observed at 1mg/l IBA.

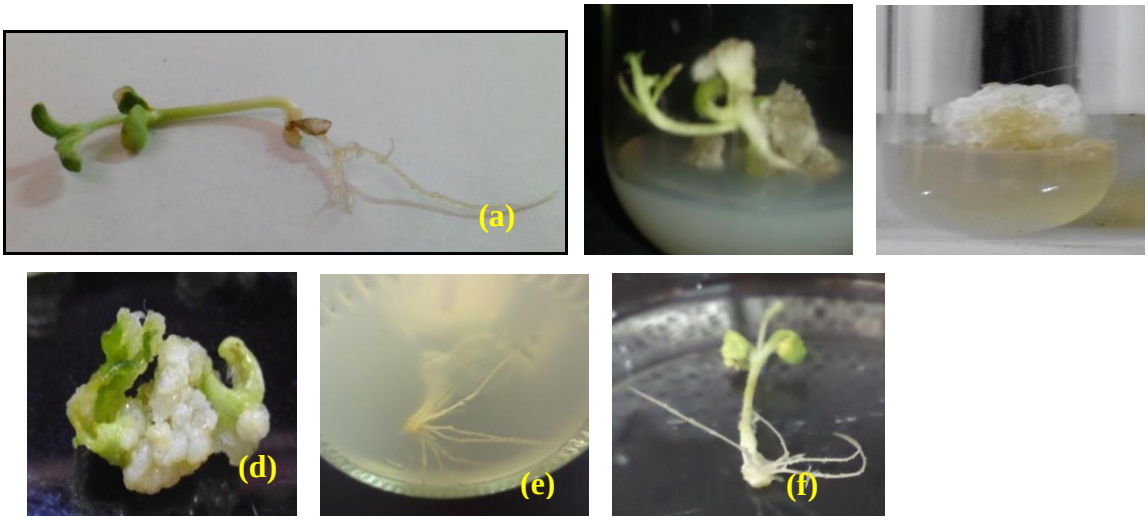

Figure 5: (a) *In vitro* seedling of *Helianthus annuus* (b) shoot induction in 0.5mg/l BAP with 0.1mg/l NAA (c), (d) callus induction in 2mg/l 2, 4 D with 0.1 mg/l BAP from the stem explants (d) the leaf explants (e) root induction in 1.0 mg/l (f) rooted sun flower *in vitro* plant

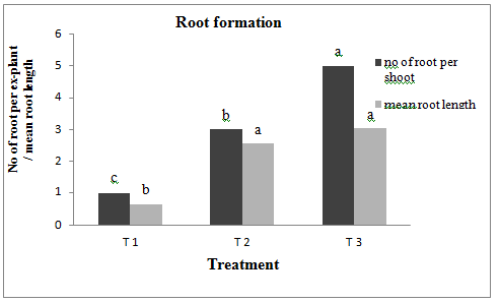

Fig. 6. Effect of IBA (mg/l) concentrations on number of root and root length
Mean with the same letters are not significantly different at P<0.05

V. Conclusion

According to results, optimum shoots induction was observed on MS basal medium containing 0.5mg/l BAP + 0.1mg/l NAA. Optimum callus induction was observed on MS basal medium containing 2.0 mg/l 2, 4 D + 0.1 mg/l BAP. Highest root formation was observed on MS basal medium containing 1 mg/l IBA. Among tested 3 types of ex-plants via stem, leaf and root; highest response for shoot regeneration and callus induction observed from stem explants in *Helianthus annuus* L.

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