INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 5, p. 85-96, 2017

RESEARCH PAPER OPEN ACCESS

In vitro organogenesis of *Ageratum houstonianum* (Asteraceae)

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Key words: *Ageratumhoustonianum,* Kin and IAA, Regeneration, Tissue culture

http://dx.doi.org/10.12692/ijb/10.5.85-96 Article published on May 14, 2017

Abstract

Regeneration of whole plants from tissue explants is a key technology for crop improvement and plant biotechnology and has critically contributed to the green revolution. In this study, the effects of MS medium supplemented with various concentrations of Kin and IAA combination on induction of callus, shoot and root induction and multiplication, and plantlet formation in various explants of *Ageratum houstonianum* were investigated. Maximum number of callus formation was observed in leaf at 1-1 and 4-4 (mg L^{-1}), and in shoot apical meristem at 2-2 (mg L^{-1}) of Kin and IAA combination. 0.4-1 (mg L^{-1}) was the best combination for root and shoot regeneration on root and leaf explants, respectively. The maximum number of both root and shoot formation in epicotyl explant was observed at 2-4 (mg L^{-1}). Among the explants, shoot apical meristems were responding for regeneration of plantlets, and the optimum concentration of Kin-IAA to produce plantlet was 4-2 (mg L^{-1}) combination. This study would be an important step towards the development of good quality of organogenesis to provide successful plantlet production from other *Ageratum* species.

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Introduction

The application of medicinal plants to maintain health and treat diseases started thousands of years ago and is still part of medical practice in many countries. *Ageratum* belongs to the family Asteraceae consists of approximately 30 species, from which a number of compounds have been reported for their medicinal and insecticidal uses (Sharma and Sharma, 1995).

Ageratum houstonianum is one of the important medicinal ornamental plants which is native to India, Central and Southern America, and has naturalized other parts of the world as weed (Kumar, 2014). A number of compounds from *A. houstonianum* have been reported (Siebertz *et al.*, 1990; Menut *et al.*, 1993; Sharma and Sharma, 1995; Wiedenfeld and Andrade-Cetto, 2001), and the plant has a long history of traditional medicinal uses. The extracts of *A. houstonianum* were reported to possess insecticidal (Ravindran *et al.*, 2012), antimicrobial (Kurade *et al.*, 2010; Tennyson *et al.*, 2011) and antifungal (Pandey *et al.*, 1984) effects. Moreover, the plant extract has anti-diabetic activity and is useful in the treatment of diabetes mellitus (Srinivas *et al.*, 2012). In addition to acaricidal properties (Tedonkeng *et al.*, 2005), the essential oil of the plant leaves contains antidermatophytic compounds that may not be toxic when used topically (Njateng *et al.*, 2010). Furthermore, *A. houstonianum* has found to be a potent source of natural antioxidants (Tennyson *et al.*, 2012) and have anticancer activity (Rizvi *et al.*, 2014).

Medicinally important plants of family Asteraceae have been successfully micropropagated through tissue culture (Tamura *et al.*,1984; Khan and Ahmad, 2008; Jain *et al.*, 2009; Das *et al.*, 2011; Amin *et al.*, 2013). However, few studies on tissue culture of *A. houstonianum* have been performed until now.

Therefore, this study provides callus induction and organogenesis characterization of various explants, includes shoot apical meristem of *A. houstonianum* to develop methods for *in vitro* propagation and to enable plantlet production.

Materials and methods

Culture media

The MS (Murashige and Skoog, 1962)basal media for seed culture and MS media supplemented with various levels of Kin (Kinetin) and IAA (Indole-3-acetic acid) combination as inducing media were prepared; The stock solutions of basic component of the media comprises of macro and micro nutrients, vitamins, 3% sucrose, 0.9% agar (Sigma Chemical Co.) and growth regulators (for inducing media) were prepared in glass distilled water. The Kin and IAA with specific concentration (0.4, 1, 2 and 4 mg L^{-1}) were initially dissolved in 1N HCl and NaOH, respectively. The media pH was adjusted to 5.7-5.8 with 0.1 N NaOH and 0.1 N HCl using the systromics pH meter before autoclaving. The media was autoclaved at 15 psi (pounds per square inch) pressure and 125 ^{o} C for 30 min [21]. Then all the stock solutions were kept in refrigerator at 4 °C.

Seed sterilization, treatment and culture

Seeds of *A. houstonianum* were obtained from Tehran parks organ green house. The seeds were sterilized by immersion in ethanol (96%) for 1 minute, then in 5% aqueous solution of sodium hypochlorite for 15 minutes.

This was followed by rinsing in sterilized distilled water at least three times. To accelerate of germination, the seeds were treated with Gibberellin (50 mg L^{-1}) for 12 hours. Seeds were cultured on hormone free MS medium.

All the process was carried out under aseptic condition in the laminar flow hood. Seeds were kept at growth chamber with specific photoperiod conditions including 12 hour light (3000 lux), at 25±2 ^{o}C. The 18-21 day-old seedlings were used as the source of explants (Fig.1).

Explants culture

Shoot apical meristem (Sm), epicotyl (Ep), root (Rt)and leaves (Lf) (blade part) segments (0.3-0.5 cm in length) were taken from seedlings. Then these explants were individually cultured on MS media supplemented with variousconcentrationsof Kin-IAA (0.4-0.4, 1-1, 2-2, 4-4, 0.4, 1-0.4, 2-4, 4-2) (mg L^{-1})combinations, in 60mm diameter Petri dishes.

All the experiments were conducted in a completely randomized design with four replicates. The cultures were maintained in a growth chamber at a constant temperature of 25±2 °C with 12 hours (light/darkness) photoperiod.

After callusing, the capable calli derived from explants were subcultured in the same media, to be evaluated in terms of organogenesis and plantlet production characterization.

The cultures were checked daily and the subsequent subcultures were done at 3 weeks intervals. The growth conditions were not altered all through the experiment. The percentage of regeneration was evaluated; the number of explants producing root, shoot or plantlet were recorded for each type of explants, after 120 days of treatment setting.

Statistical analysis

The data were analyzed by one way ANOVA and Tukey Post Hoc. Data were expressed as mean±SE. Duncan's multiple range test (at $p < 0.05$) was employed for the mean comparisons. Statistical analyses were performed using Excel software and SPSS (18.0) for Windows 2007.

Results and discussion

Effect of different concentrations of Kin-IAA combination on callus induction

In this study, explants showed marked difference in their callus induction days, tocallus size andgrowth rate. Generally the callusing response was initiated by thickening and swelling, especially from the cut end of the explants after one week of culture.

The callus tissues was compact and dark yellow-light brown in color (Figs 2, 3, 4).Also, the amount of callus production and survival of the calli was varied depending on the type of explants and the concentrations of Kin-IAA combination (Data not shown).

The rate of growth callus produced from the most explants was low. However, Lf and Sm showed superiority over the other types of explants in callusing and supported better growth (Figs 2A, B, 5D)which is in the agreement with previous studies (Abou-Mandour, 1999; Anbazhagan *et al.*, 2010; Amin *et al.*, 2013).

Fig. 1. Seedlings; Seed germination in free hormone MS basal media.

According our study, 1-1 and 4-4 (mg L^{-1}) were found to be the optimal concentrations of Kin-IAA for callus production in leaf samples (Figs 2A, B).

Ep and Rt calli were slow growing and had a low tendency to form callus (Figs 3, 4). Moreover, a longer exposure of calli to media resulted in growth inhibition, even at low concentrations (Figs 2C-G, 3, 4, 5A-F). Regard to this result, there aremany studies that support NAA as the best suited for callus producing and continuous growing among the various auxins tested in Asteraceae family; *Momordicadioica*(Nabi *et al.*, 2002),*Rauwolfia serpentina* (Tomarand and Tiwari, 2006),*Asteracantha longifolia* (Panigrahi *et al.*, 2006),*Adhatoda vasica*(Sarin and Bansal, 2011) and *Helianthus annuus*(Nokhasi *et al.,* 2014).

This may explain the reason of low growth rate of calli in this research.

Also, Kin did not prove to be beneficial for producing healthy callus. Some authors suggest that callus can be produced without cytokinin in the medium (Del Pozo *et al.*, 2005; Gordon *et al.*, 2007; Atta *et al.*, 2009).

Other hand, Sm explants produced callus in all treatments, except 4-4 and 0.4-1 (mg L^{-1}). 2-2 (mg L^{-1}) combination was best for callus induction and establishment. The calli so produced were compact and possessed high capacity of growth (Fig. 5D).

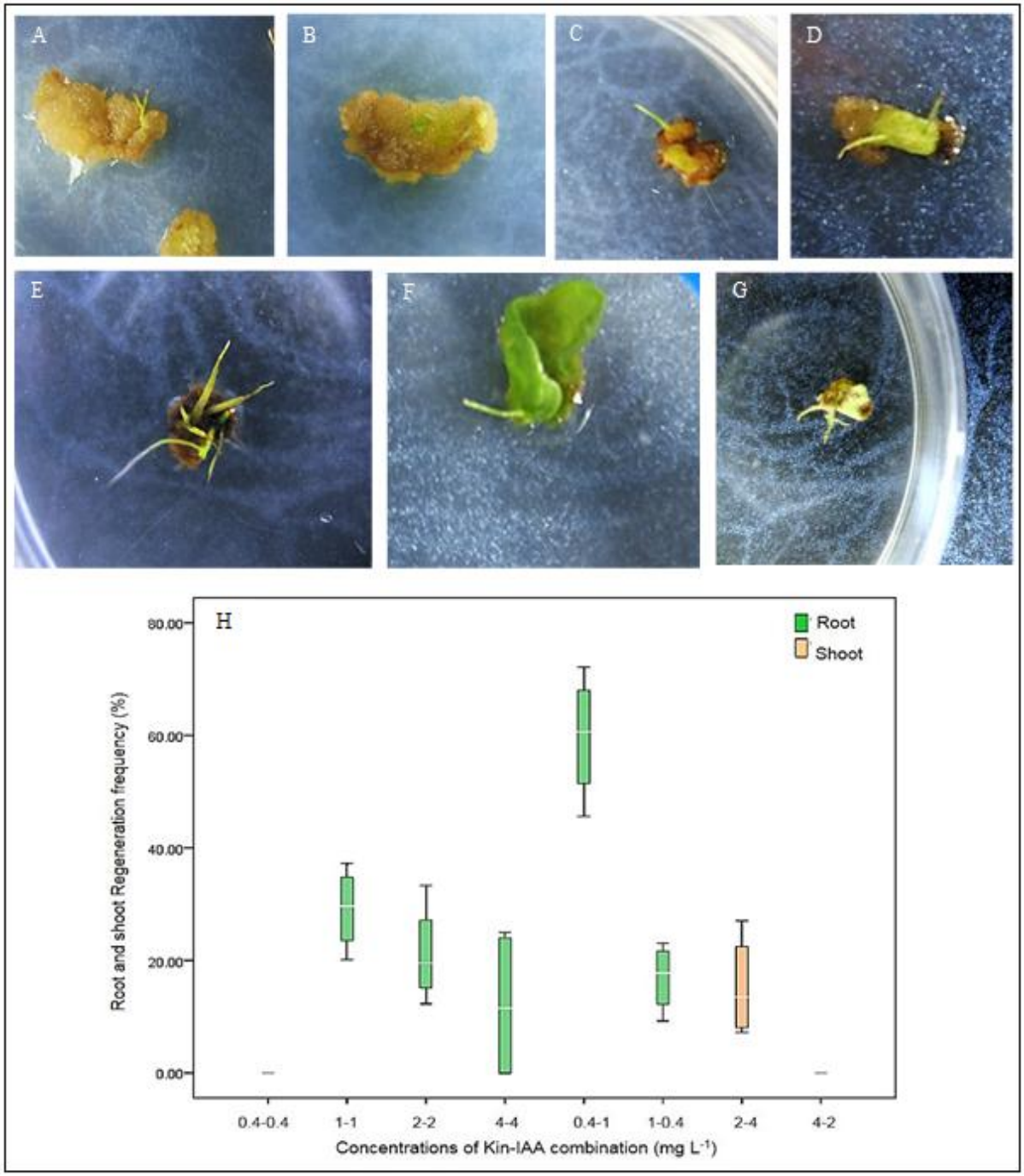

Fig. 2. Callus induction and Organogenesis from leaf explants in different concentrations of Kin-IAA (mg L-1) combination. A: callus formation and shoot regeneration in 1-1 (mg L-1), B: Effective callus formation in 4-4 (mg L-1), C: Shoot regeneration in 4-4 (mg L-1), D: Shoot regeneration in 2-2 (mg L-1), E: Shoot regeneration in 0.4-1 (mg L-1), F: Shoot regeneration in 1-0.4 (mg L-1), G: Regeneration of roots in 2-4 (mg L-1), H: Comparison of different concentrations of Kin-IAA (mg L-1) effect on root and shoot regeneration from leaf calli ($n = 4$) ($p < 0.05$).

Effect of different concentrations of Kin-IAA combination on shoot and root regeneration

The calli of Lf, Ep and Rt which had better potential of being differentiated, showed some degree of organogenesis, that was found to be hormone dependent.

Leaf explants

The shoots regenerated from leaf calli were slow growing and showed retarded growth within 2 weeks (Figs 2A-F). The highest amount of shoot regeneration with the mean of 59.75% ±5.97 ($P<0.05$) was achieved at 0.4-1 (mg L^{-1}) (Figs 2E, H).

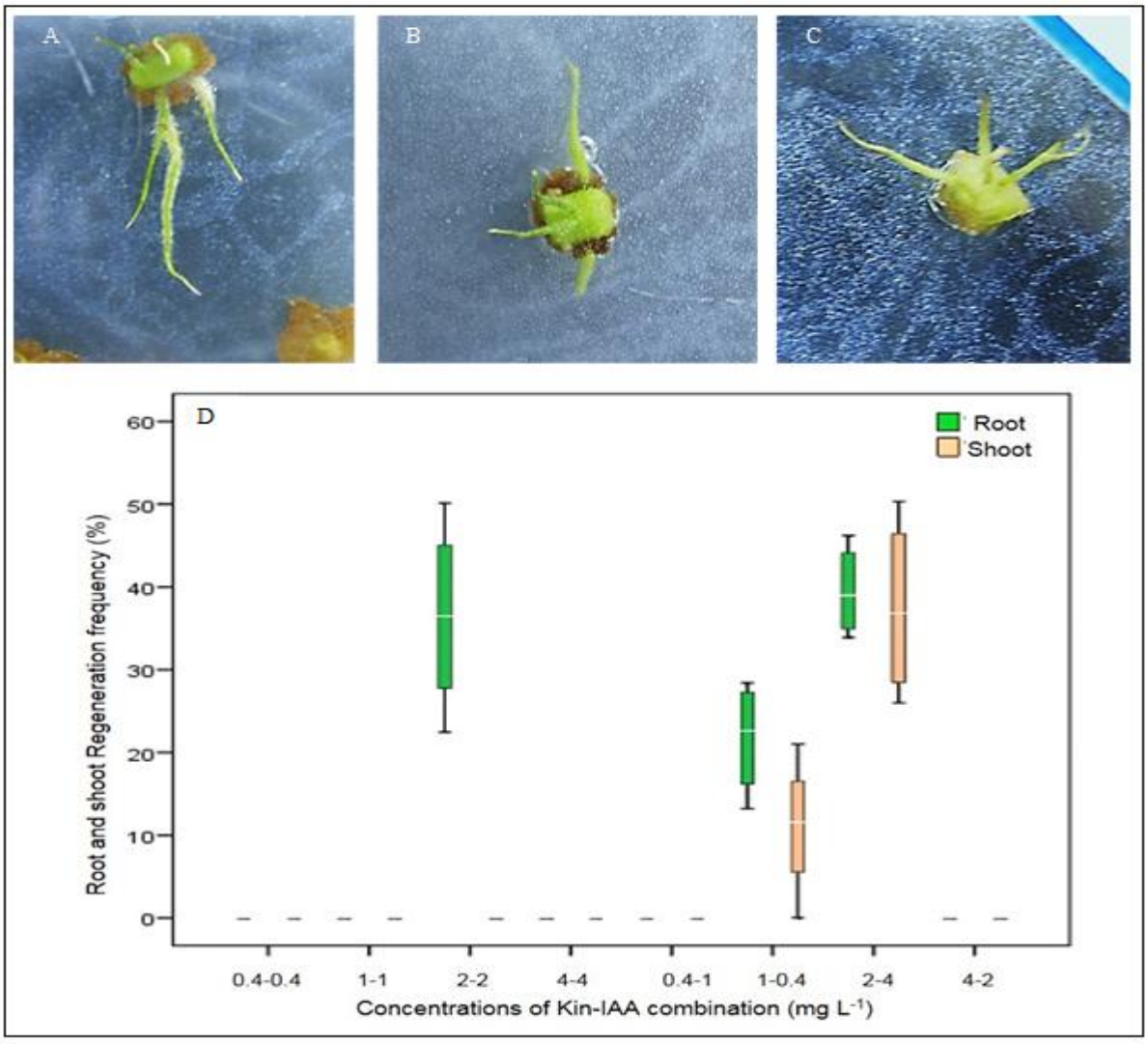

Fig. 3. Callus induction and Organogenesis from Epicotyl explants in different concentrations of Kin-IAA (mg L-1) combination. A: Root and shoot regeneration in 2-4 (mg L-1), B: Shoot and root regeneration in 1-0.4 (mg L-1), C: Shoot regeneration in 2-2 (mg L-1), D: Comparison of different concentrations of Kin-IAA (mg L-1) effect on root and shoot regeneration from epicotyl calli (n = 4) ($p < 0.05$).

Root regeneration on leaf calli was observed only at 2-4 (mg L^{-1}) with the mean amount of 15.3% ±2.09 ($P<0.05$) (Figs 2G, H).

Epicotyl explants

Regenerated shoots on Ep calli showed very slow growth and reduced elongation, in 2-2, 1-0.4 and 2-4 (mg L^{-1}) of Kin-IAA combination (Figs 3A-C). However the number of shoots was higher in 2-2 (mg L^{-1}) treatment (Fig. 3C). Ep explants showed the emergence of hairy roots at 2-4 (mg L^{-1}) and a relative potential of rooting at 1-0.4 (mg L^{-1}) with the mean amount of 37.47%±3.49 and 11.2%±3.49 ($P<0.05$), respectively (Figs 3A, B, D).

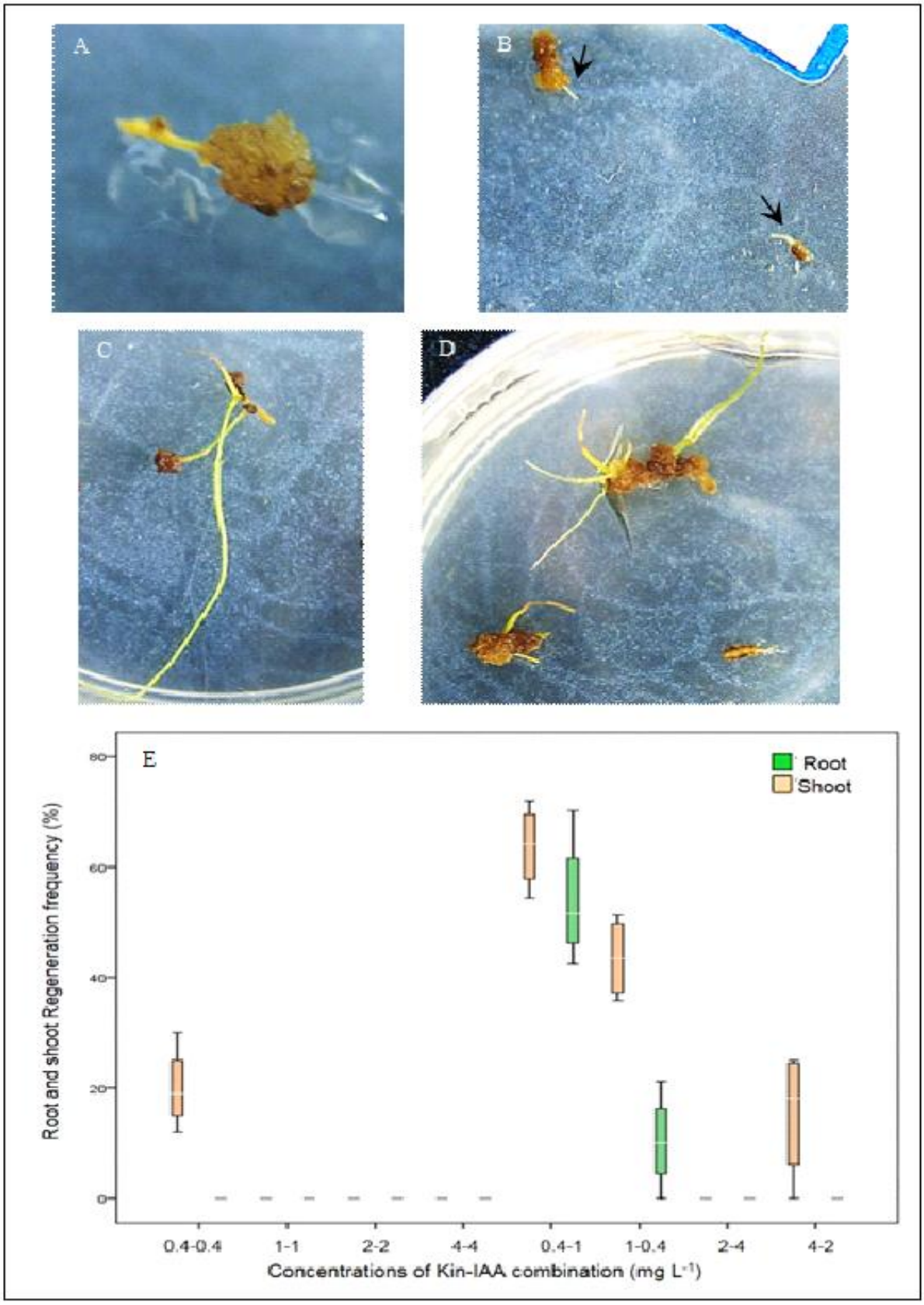

Fig. 4. Callus induction and Organogenesis from root explants in different concentrations of Kin-IAA (mg L-1) combination. A: Root regeneration in 0.4-0.4 (mg L-1), B: Root regeneration in 4-2 (mg L-1), C: Root and shoot regeneration in 1-0.4 (mg L-1), D: Root and shoot regeneration in 0.4-1 (mg L-1), E: Comparison of different concentrations of Kin-IAA (mg L-1) effect on root and shoot regeneration from root calli (n = 4) ($p < 0.05$).

Root explants

In the Rt explants, the root regeneration potential in 0.4-0.4 and 4-2 (mg L^{-1}) was low (Figs 4A, B). In 1-0.4 and 0.4-1 (mg L^{-1}) combinations, relative fast growing roots were regenerated, and shoot formation was observed (Figs 4C, D).

0.4-1 (mg L^{-1}) combination enhanced the response of calli for root and shoot formation with the mean amount of 63.65±4.37 and 53.95% ±3.64 ($P<0.05$), respectively, although the growth of shoots was arrested within 10 days of regeneration (Figs 4D, E).

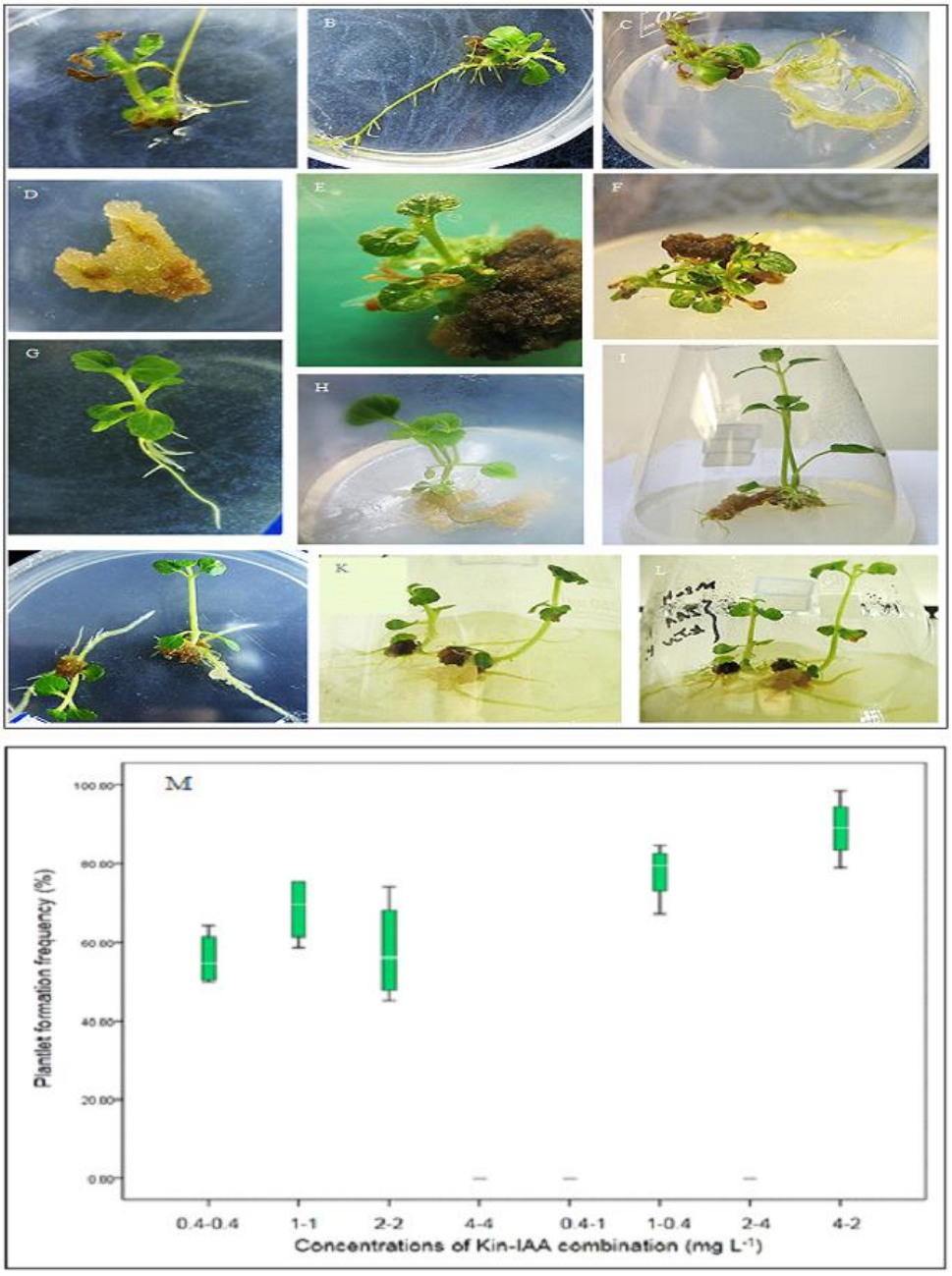

Fig. 5. Successful *in vitro* culture of *Ageratum houstonianum* from the shoot apical meristem explants in different concentrations of Kin-IAA (mg L-1) combination. A: plantlet formation in 0.4-0.4 (mg L-1), B: plantlet formation in 1-1 (mg L-1), C: b after 20 days, D: Effective callus formation in 2-2 (mg L-1), E: D; leaved shoot formation after 120 days, F: plantlet formation in 2-2 (mg L-1) after 5 months, G: plantlet formation in 1-0.4 (mg L-1) after 45 days, H and I: G after 15 and 30 days, respectively, J and K and L: plantlet formation in 4-2 (mg L-1), M: Comparison of different concentrations of Kin-IAA (mg L-1) effect on root and shoot regeneration from shoot apical meristem calli ($n = 4$) ($p < 0.05$).

Our results revealed that the highest number ofshoot and shooting percentage among mentioned explants were observed in Lf (59.75%±5.97), followed by Rt explant (53.95%±3.64), in 0.4-1 (mg L^{-1}) Kin-IAA combination (Figs 2H, 4E).These results showed that shoot regeneration frequencydecreased with the ascending concentrations of Kin, then increased when higher amount of IAA (1 mg L^{-1}) wasapplied. Similarly, shoot regeneration from Ep explants was observed in 2-4 (mg L^{-1}) combination (Fig. 3D). These results show the stimulating effects of IAA treatment on effective shooting in the agreement of previous studies on Asteraceae family (Hwang, 2006; Senthilkumar and Paulsamy, 2010; Paulsamy and Ganesan, 2011).

Also, direct differentiation of shoot was also obtained (<20%) from leaf explants in 1-0.4 (mg L^{-1}) of Kin-IAA combination (Fig. 2F). This result is in the agreement of previous studies (Juan *et al.*, 2006; Jain *et al.*, 2009; Yarra *et al.*, 2010) in which higher levels of cytokinin relation to auxin triggered the direct shoot regeneration response from Lf explants.

Root regeneration from Lf calli on 2-4 (mg L^{-1}), and regeneration of fast growth green hairy roots from Ep and Rt calli on 2-4 (mg L^{-1}) and 0.4-1 (mg L^{-1}), respectively, was observed (Figs 2G, 3A, 4D) which is due to the use of higher level of IAA relative to Kin. This result is similar with previous study (Pinto José *et al.*, 2007) in which high levels of auxin concentration could influence rooting success in *Lychnophora pinaster*. In fact, exogenous auxin treatment, as well as its normal transport and accumulation during root development, induce root formation (Himanen *et al.*, 2002; Laskowski *et al.*, 2008).

However, relative root regeneration was observed from Ep explants (11.02%± 3.49) in 1-0.4 (mg L^{-1}) ratio (Figs 3B, D), which is probably a result of sufficient free auxin level in their tissues (Xu *et al.*, 2014). Besides, low ability of Lf in root regeneration on other Kin-IAA ratios is probably a result of insufficient free auxin level in their tissues (Fig. 2H). In the consistent with this idea, it is important to use younger leaves with higher endogenous auxin levels for efficient regeneration of roots (Shoji *et al.*, 1951; Xu *et al.*, 2014).

Organogenesis and plantlet production from shoot apical meristem (Sm)

In 0.4-0.4 (mg L^{-1}), regeneration of root and shoot followed by plantlets formation with the amount of 55.87% ±4.88 ($P<0.05$). However, the plantlets (2-3 cm height) showed leaf necrosis and did not survive (Figs 5A, M).

In 1-1 (mg L^{-1}), plantlet production with fast growing root and high number of leaved shoot were observed after 45 days of culture (66.77±4.88, $P< 0.05$) (Figs 5B, M). But the leaves turned brown and the shoot showed retarded growth and was incapable for further growth, after 2-3 weeks (Fig. 5C).

Likewise, the concentration of 2-2 (mg L^{-1}) induced root and shoot regeneration after 100 days of culture (Fig. 5E). Plant production was totally observed after 5 months (57.85±4.88, $P< 0.05$) (Figs 5F, M). The plantlets were bulkier, in spite of slow growing. The leaves were trichomic and relatively dentate. The growth of the plantlets was gradually diminished because of degeneration of the calli (Figs 5D-F).

The 1-0.4 (mg L^{-1}) had the efficiency of initiation for organogenesis and plantlet production, within 42-51 days at high percentage of Sm explant (77.62% ±4.88, $P<0.05$) (Fig. 5M). The higher shoot length of 12cm was achieved and the leaves were healthy and dentate, but the growth of plantlet was slow (Figs 5G-I).

The most frequent induction of roots and shoots in Sm calli was observed in 4-2 (mg L^{-1}) combination, and it was proved to manifest the highest percentage of plantlet formation (88.77±4.88, $P< 0.05$) (Figs 5J, M). The root length was greater (3.0cm) than the ones generated on 1-0.4 (mg L^{-1}) media, and the number of roots was also observed to be higher (Figs 5K, L).

Moreover, the plantlets were bulkier and the growth rate was considerable. The leaves gradually showed necrosis within 3-4 weeks of generation (Figs 5J-L).

The 2-4 (mg L^{-1}) combinations initiated only slow growing small calli and no organogenesis was observed (Data not shown). The results of this study showed that the doubled amount of Kin than IAA in comparison with same levels of Kin and IAA combination, enhanced the response of Sm calli for producing plantlets (Fig. 5M).

According our results, among the various explants tried, the Sm explants proved to be the best for organogenesis, which followed by plantlet production. The root and leaved shoot obtained, synchronously, were fast growing and healthier than the ones derived from other explant calli. Plantlet formation in Sm explants reveals the superiority of Sm over the other types of explants in organogenesis potential (Figs 5). This is in general agreement with previous studies (Anbazhagan *et al*., 2010) that reported the best plantlet regeneration was obtained from *Stevia rebaudiana*by using shoot tips, and Kin and IAA were of the most effective regulators for inducing organogenesis.

Our results also indicated that significantlyhigher plantlets were produced in 4-2 (mg L^{-1}) (88.77%±4.88) and 1-0.4 (mg L^{-1}) (77.62% ±4.88) Kin-IAA combinations, while by employing double levels of IAA than Kin, no organogenesis from Sm explants was observed (Fig. 5M). This result is consistent with studies (Tamura *et al*., 1984; Das *et al*., 2011), in which they achieved *Stevia rebaudiana* micropropagation through shoot tip culture by using high concentration of kinetin in medium, and reported that media supplemented with IAA had an adverse effect on root induction.

Although it sounds that high levels of Kin in culturemedium could confer beneficial effects to plantlet formation from Sm, same amounts of Kin and IAA (0.4-0.4, 1-1 and 2-2 (mg L^{-1})) also lead to plantlet production and multiplication (Figs 5A, B, F). However, medium supplemented with higher amount of Kin resulted in elongated shoots in this research (Figs 5G-L). This results is similar with previous studies (Sujatha and Kumari, 2007; Kumar and Kumari, 2010) in which the significant effect of IAA and the influence of Kincombined with otherphytohormones on *in vitro*micropropagation of *Artemisia vulgaris,* using its shoot tip, has been reported.

During this study, we frequently observed that the regenerated roots had the ability to become green (Figs 3A, B; 4C, D; 5A, B, F, G, J), in spite of light-dark photoperiod condition. This result is in the agreement with Flores studies (Flores *et al*., 1988; Flores *et al*., 1993)in which the potential for greening and photosynthetic ability by many root cultures of Asteraceae genera has been reported. Likewise, regeneration of green roots has been reported before (Abou-Mandour, 1999) in a tissue culture investigation of *Ageratina riparia*. It is interesting that roots express their capacity for carbon autotrophy, which provides new ways for manipulating and utilizing the vast biosynthetic potential of roots (Flores *et al.,* 1993).

Conclusion

The results of this study showed that we can activate system of root and shoot regeneration for *A. houstonianum,* using the suitable explants and effective combination of hormones auxin and cytokinin. Moreover, we can obtain samples with high performance of regeneration that they have ability to become a complete plant.

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