Evaluation of callus induction and plant regeneration in *Citrullus colocynthis* (L.) Schrad

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Abstract

In the present study, small segments of cotyledon, apical bud, hypocotyl, and root of 10-day-old seedlings were isolated and transferred to Murashige and Skoog (MS) base medium with different treatments of phytohormones. Two media were used for apical bud culture: MS with indole-3-acetic acid (IAA) and kinetin (Kin) (1 mg/l) and the other medium, MS with double vitamin of MS and 6-benzylaminopurine (BAP) (2 mg/l) and naphthalene acetic acid (NAA) (0.1 mg/l). The findings suggested that capacity of callus generation in BAP and NAA was better than IAA and Kin, but shoot generation and increase of shoot length under IAA and Kin treatment were significant. In BAP and NAA media the callus differentiated in shoot, but generation of shoot and increase in its length was not significant.

Keywords: Citrullus colocynthis; Indole-3-acetic acid; Kinetin; Naphthalene acetic acid; 6-Benzylaminopurine

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Introduction

Citrullus colocynthis belongs to the family Cucurbitaceae. This plant is commonly known as bitter apple or bitter cucumber (AbdelHassan et al., 2000). It is native to warmer parts of Asia, Syria, Egypt, and Marine region of the Mediterranean (Memon et al., 2003). *Citrulls colocynthis* is a small perennial creeping herb with prostrate or climbing stem, bearing smooth spherical fruits which are mottled green when young and somewhat yellow when ripe (Shah and Qadry, 1985).

For centuries, humankind has been totally dependent on plants as source of carbohydrates, proteins and fats for food, and shelter. In addition, plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, flavors, fragrances, colors, biopesticides, and food additives (Rao and Ravishankar, 2002). *C. colocynthis* leaves contain cucurbitacin A, B, C, and D, α-elaterin, and probably other constituents (Tanninspitz et al., 2007; AlYahya et al., 2000).

It is estimated that, 75% of the world's population relies on plants for traditional medicine (Rao and Ravishankar, 2002). This establishes the importance of *Citrullus colocynthis* as it is used in folk medicine by people in rural areas as a purgative, anti-rheumatic, anti-diabetic, and also as a remedy for skin infections (Tanninspitz et al., 2007). Being a source of substances with anticancer properties, this plant also plays an important role in cancer

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Table 1
Callus and organogenesis induction media used in the experiments.

Media codes	Basal Medium	Vitamin	IAA (mg/l)	NAA (mg/l)	BAP (mg/l)	Kin (mg/l)
A	MS	MS	1.0	-----	----	1.0
C	MS	MS	-----	------	-----	6.0
E	MS	MS	-----	----	2.5	-----
F	MS	MS	------	0.4	------	0.4
I	MS	2 MS	------	0.1	2.0	------
M	MS	MS	1.0	-----	-----	-----

*Murashige and Skoog (1962).

treatment (Tanninspitz et al., 2007). In addition, immature fruit and seed extracts showed a broad spectrum of antimicrobial activities (Marzouk et al., 2011).

Regeneration of bitter apple and cucumber plants has been reported on various explants. In *C. colocynthis*, these include shoot tip culture (Meena et al., 2010), leave, and stem (Savitha et al., 2010). In *Cucumis sativum*, explants involved leaves (Usman et al. 2011), cotyledon, and hypocotyls (Ugandhar et al. 2011). Moreover, amino acids and polyamines are reported to enhance multiple shoot formation from shoot tip explants of cucumber (Vasudevan et al., 2008). In *Cucumis melo* var. *flexousus,* the best regeneration capacity of cotyledon explants has been obtained from the seeds germinated under dark condition rather than the light one (Mendi et al., 2010). Also regeneration via indirect organogenesis from internodal explants of bitter melon (*Momordica charantia* L.) has been reported by Thiruvengadam et al. (2012).

The main goal for our study was obtaining isolated tissue culture of *C. colocynthis* using sterile seedlings. The paper also reports *in vitro* callus formation, growth of callus, and organogenic response of *C. colocynthis*, to determine the optimal media and select the appropriate explants. Since the method of isolated culture allows regulation of synthesis of secondary metabolites, it would be useful to introduce this plant for *in vitro* cultivation. An *in vitro* propagation protocol can provide plant material for future pharmacological, physiological, and biochemical studies.

Materials and Methods

Two sequential experiments were carried out. These are explained as Experiments 1 and 2 in the following sections.

Experiment 1. Effect of explants source and the medium culture on callus induction and organogenic response

Seed dormancy was removed with gibberellic acid (10 mg/l) treatment for 24h. After a two - stage sterilization, the seeds were grown on MS medium without any growth regulators. 10-day-old seedlings were cut into roots, hypocotyls, cotyledons and apical buds, and these were used as explants. Subcultures were continued every 28 days and the callus was kept under darkness at 30 °C.

All the media contained 3% (w/v) sucrose. The pH of the media was adjusted to 5.7 and solidified with 0.8% agar before autoclaving at a pressure of 15 psi and temperature set at 121 °C for 20 min. The culture media used for callus and organogenesis induction are described in Table 1.

Experiment 2. Shoot generation and their length

Two media were used for apical bud culture (A and I). The experimental design was completely randomized with 10 replications. Cultures were maintained in a growth room at 30 °C under 16/8- h (light/dark) photoperiod. The explants were scored with the aid of a stereomicroscope after 14, 21, and 35 days. Frequency of callus induction from explants, number of shoots per responsive explants, and

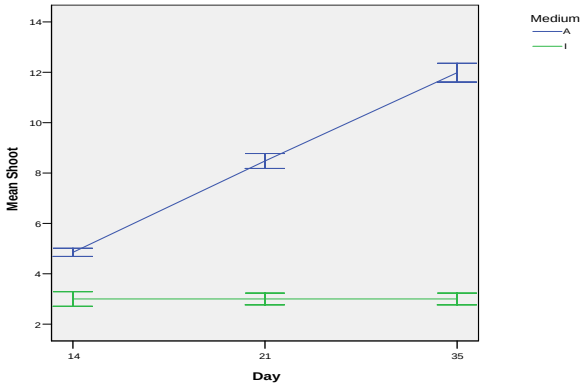

Fig. I. The effect of culture medium (A & I) on number of shoots per apical bud

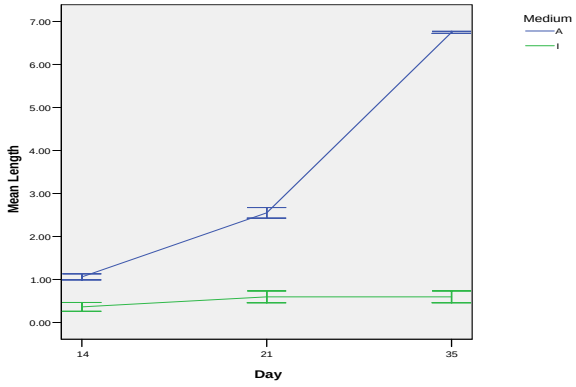

Fig. II. The effect of culture medium (A & I) on length of shoots (cm) per apical bud

length of shoots were recorded. ANOVA was used as the statistical using SPSS and differences among means were tested by Duncan range test ($p < 0.05$).

Results

The choice of appropriate explants constitutes a main step in the establishment of plant regeneration protocols. Callus was obtained from all types of explants. For all explant sources taken together, the efficiency of adventitious bud formation was noted in apical bud explants on two medium combinations: MS medium supplemented with 2.0 mg/lBAP + 0.1mg/l NAA + double vitamin of MS and MS medium supplemented with 1.0 mg/l Kin, 1.0 mg/l IAA. The apical bud cultured on MS medium with different concentrations of growth regulators were enlarged in size within 14 days after culture in all the treatments. In the treatments with IAA and Kin 1.0 mg/l of each, shoot buds appeared directly from the apical bud explants after 8 - 10 days, and the axillary shoots proliferated and elongated by 0.5 - 1 cm within 14 days. The longest shoots (6.69 ± 0.30) and the highest number of shoots per apical bud (11.81 ± 0.57) were observed after 35 days of the culture. The shortest shoots (0.3 ± 0.09) and lowest shoots formation (2.14 ± 0.44) were noted in the MS medium containing double vitamin of MS + 2.0 mg/lBAP + 0.1 mg/l NAA after 35 days of culture (Figs. I & II).

The results from two medium combinations and apical bud together indicated the highest percentage of callus growth (60%) obtained on MS medium with double vitamin of

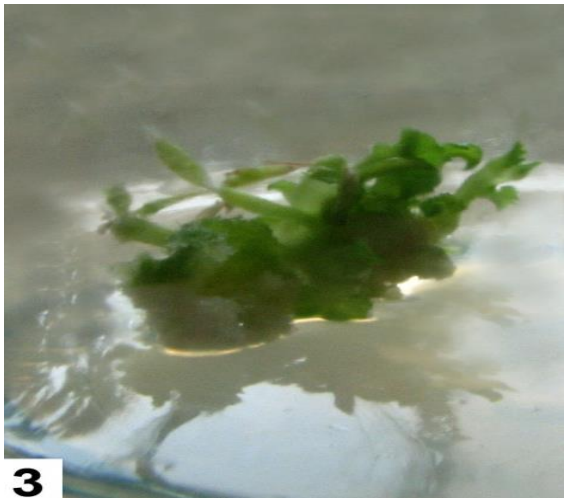

Fig. III. Adventitious shoot regeneration from apical bud explant on MS medium supplemented with 2mg/l BAP and 0.1mg/l NAA+2MS vitamin, after 35 days

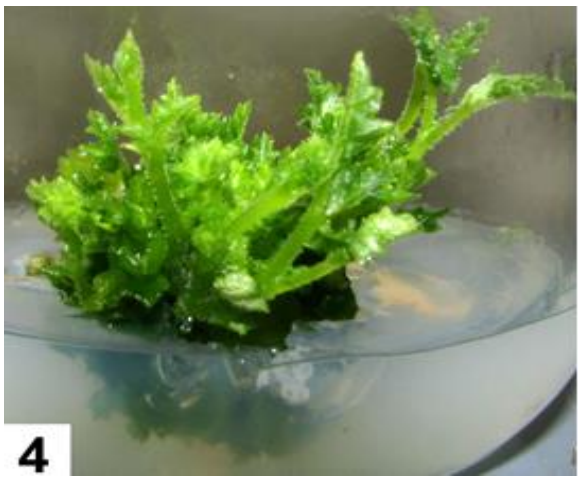

Fig. IV. Shoot regeneration from apical bud explant on MS Medium supplemented with 1mg/l Kin and 1mg/l IAA after 35 days

MS + 2.0 mg/lBAP + 0.1 mg/l NAA. The evaluated culture media showed significant difference on organogenesis (Figs. III & IV).

For root formations, 2 - 2.5 cm-long shoots were transferred to rooting medium consisting of MS basal medium supplemented with 1 mg/l IAA (Fig. V). Rooted plants were transferred to soil and grown in a greenhouse (Fig. VI).

Discussion

Effects of various concentrations of plant growth substances were studied on callus induction and plant regeneration from different explant sources. All the combination of hormones induced callus on the basal part of the explants, as reported by Chaturvedi et al. (1984) for *Costus speciosus* shoot-tip explants. Ugandhar et al. (2011) reported about enhancement of shoot formation by proliferation of hypocotyl and cotyledon explants from *Cucumis sativum* (L.) on a medium fortified with cytokinin and auxin. There was no shoot formation on callus from hypocotyl and cotyledon explants in the present study.

The calluses derived from root explants were best for differentiation to root on MS medium supplemented with 1.0 mg/l Kin, 1.0 mg/l IAA, after 3 weeks. In contrast to the above mentioned finding, Turker et al. (2008) observed that the best shoot proliferation of *Epilobium angustifolium* L. was obtained from root explants cultured (direct organogenesis) on media with 0.1 mg/l BA and 0.5 mg/l IAA.

Apical bud collected from explants responded with adventitious bud formation as compared with explants collected from either roots or hypocotyls. Medium combination and explants origin had important effects not only on callus initiation but also on plant regeneration. Similar results were also observed in organogenesis of watermelon (Krug et al., 2005) and melon (Souza et al., 2006).

Moreno et al. (1985) tested several culture media with different concentrations of IAA and Kin in Amarillo Oro cultivar (melon). The most effective combination for organogenic response was 1.5 mg/l of IAA and 6.0 mg/l of Kin. Dong and Jia (1991) reported an improvement in shoot bud development in watermelon cotyledons when combining cytokinin and auxin in the induction media, but both Compton and Gray (1993) and Srivastava et al. (1989) detected

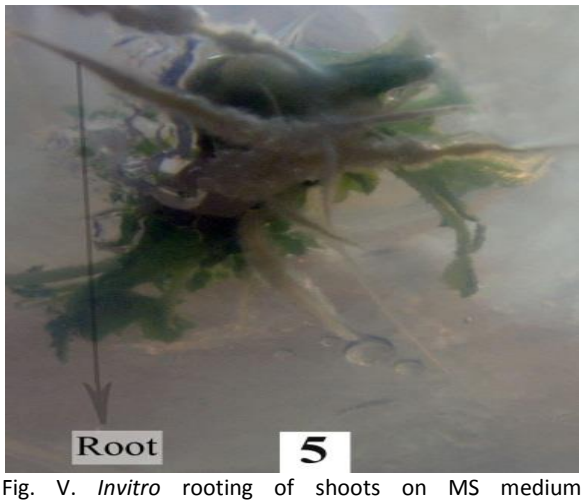

Fig. V. *Invitro* rooting of shoots on MS medium supplemented with 1.0 mg/l IAA

Fig. VI. Regenerated and hardened plantlet

an inhibition of shoot organogenesis when NAA or IAA were added to the induction medium.

The composition of induction media is also important for adventitious bud development; the presence of cytokinin is critical for shoot induction in watermelon (Dong and Jia 1991). Moreover, combination of cytokinin favored multiple shoot proliferation in *Ocimum sanctum* (Girija et al. 2006) *C. annuum* (Rao et al., 2006). However, for the number of shoots per apical bud, MS medium with IAA and Kin 1.0 mg/l of each, favored the development of buds, resulting in a higher number of shoots (11.81 ± 0.57).

Meena et al. (2010) reported that MS medium with 1 mg/l BAP and 1 mg/l NAA produced frequency shoot regeneration (16.8 ± 0.180) from shoot tip explants of *Citrullus colocynthis.* Differences in promotion of adventitious shoot proliferation may be due to

the brand of cytokinin and auxin or for the type of explant application.

Addition of low concentration of NAA (0.1 mg/l) to the medium containing relatively high concentration of BAP (2 mg/l) did not improve the rate of shoot proliferation (2.14 ± 0.44) in *Citrullus colocynthis.* Meena et al. (2010) reported that MS medium with 2 mg/l BAP only and MS medium with 2 mg/l BAP and 2 mg/l NAA produced shoot regeneration (11.2 ± 0.180) and (12.8 ± 0.336), respectively. In the present study, the factor inhibiting adventitious shoot proliferation may be attributed to the double dosage of vitamins in the MS medium.

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