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Plant Regeneration from Cotyledon Explants of Bittergourd as influenced by Adenine Sulphate and D-Biotin

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ABSTRACT

The first experiment was done to select the most suitable explant to establish a culture for shoot regeneration. Four different explants (cotyledon, cotyledon node, leaf and hypocotyl) excised from *in vitro* grown seedlings were cultured on MS medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. Cotyledon was the most responsive explant which exhibited quick callus initiation. The second experiment was conducted to determine the effect of cotyledon explants on different MS media containing 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and also various concentrations (0, 20, 40 and 60 mg l^{-1}) of adenine sulphate (AS). The best medium for callus formation was MS medium with 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 60 mg l^{-1} AS. Callus derived from the particular medium also showed the best positive response for shoot regeneration after subculturing on MS medium with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. In the third experiment, D-biotin added into the composition of the culture medium had a beneficial effect on the callogenesis in association with BAP and NAA. Regenerated shoots were multiplied on MS medium with 3.0 mg l^{-1} BAP. Eventually, *in vitro* rooting was achieved on MS medium included with 1.0 mg l^{-1} IBA.

Keywords: Adenine sulphate, Bitter gourd, Cotyledon, D-biotin, *In vitro* culture, Plantlets.

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INTRODUCTION

Bitter gourd (*Momordica charantia* L.) belonging to the family Cucurbitaceae is cultivated throughout the world. It is a good source of iron, calcium, phosphorus vitamin B and it contains good dietary fiber levels (Sultana and Miah, 2003). Bitter gourd seeds have very effective antiviral, antibacterial and medicinal properties (Beloin *et al*., 2005). In Sri Lanka, bitter gourd is eaten as a vegetable and it is commonly propagated by seed. Under biotic stress, varietal improvement of bitter gourd is needed to produce superior varieties. *In vitro* culture technique is an important role in plant

propagation. Successful plant propagation of bitter gourd depends on many factors in which types of explants and culture medium are significant factors in mass plant propagation. *In vitro* plants have been regenerated through organogenesis in cotyledonary explants of *Momordica charantia* L. (Malik *et al.,* 2007; Wang *et al.,* 2008; Ma *et al.,* 2012). In general, high cytokinin and low auxin are widely used plant growth regulators for plant regeneration under *in vitro* conditions.

Agarwal and Kamal (2004) noted shoot differentiation of *Momordica charantia* in the culture medium supplemented with BAP alone. Benzyladenine (BA) is important for shoot bud formation of bottle gourd (Saha *et al*., 2007). Samantaray and Maiti (2011) stated that adenine sulphate is useful in initiation of multiple shoot buds in *Chlorophytum arundinacearum*. Seran *et al*. (2006) reported that somatic embryos were formed in leaf callus cultured in MS medium with BAP (1.0 mg l^{-1}), NAA (0.1 mg l^{-1}) and adenine sulphate (0.1 mg l^{-1}). George *et al*. (2008a) also mentioned the benefits of adenine together with benzylaminopurine (BAP) in plant propagation. Vitamins are necessary compounds in plant cell in tissue culture (Diab, 2015). The addition of vitamins in the culture medium has effect on callus induction (El-Shiaty *et al*., 2004) and somatic growth (Abrahamian and Kantharajah, 2011). In the present study, the effects of adenine sulphate and D- biotin in combination with BAP and NAA were assessed for *in vitro* organogenesis through callus stage of bitter gourd.

MATERIALS AND METHODS

This study was carried out at the Tissue Culture Laboratory of the Department of Crop Science, Faculty of Agriculture, Eastern University, Sri Lanka. Seeds of bitter gourd *cv* thinnaveli white were obtained from the Department of Agriculture, Gannoruwa, Sri Lanka. The seeds were decoated and cleaned by washing in running tap water for 30 min. They were then surface sterilized with 70% (v/v) ethanol for 60 sec followed by treatment with 20% (v / v) Clorox ™ (sodium hypochlorite) solution for 20 min. And then they were rinsed three times in sterile distilled water to remove traces of sodium hypochlorite. The sterilized decoated seeds were divided into two portions and then embryogenic portions were placed on

hormone free MS (Murashige and Skoog, 1962) medium for germination. They were grown at 25 ± 2 ^{0}C under 16/8 h (light/dark) photoperiod provided by cool white fluorescent tubes with 2300 ± 200 lux intensity and relative humidity of 60 - 70% to obtain *in vitro* plantlets. The basal medium used in this investigation consisted of MS salts supplemented with 3% sucrose (Analytical grade, Himedia, India), and 0.8% agar (Bacteiological grade, Himedia, India). The pH of all media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl before solidification.

Explant Selection

It was done to select the most responsive explants for best morphogenic response under *in vitro* conditions. Leaf (5 - 6 mm^2), cotyledon (5 - 6 mm^2), cotyledon node (12 - 15 mm) and hypocotyl (3 - 5 mm) segments were excised from the 12 days old *in vitro* seedlings (Figure 1) and used as explants. Excision of the explants from *in vitro* seedlings was done under aseptic conditions by using sterilized sharp scalpel.

The excised explants were cut into appropriate size and then they were inoculated on MS basal medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. After inoculating the explants, culture bottles were tightly capped, sealed and transferred to growing area. Days taken for callus induction, callus formation %, callus weight and morphogenic response of the explants were recorded at the regular intervals. There were three replicates for each treatment and each replicate contained twelve explants.

Figure 1. *In vitro* seedling to excise the explants.

Addition of Adenine Sulphate for Cotyledon Explant Cultures

This experiment was carried out to determine the effect of adenine sulphate (AS) on the cultured cotyledon explants as most responsive explant which was selected in the previous experiment. Cotyledons were

dissected into four quarters and sterilized. Then explants were inoculated in the culture bottles containing 10 ml MS medium with 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and different concentrations of AS according to Table 1 and they were placed under *in vitro* conditions. Subsequently, they were regularly observed for any *in vitro* response. This experiment included four treatments with three replicates. Twelve explants were used for each replicate. Data were recorded at regular intervals. After six weeks of culture, subculturing was done on MS medium with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA for shoot regeneration.

Table 1. Different MS media combined with Adenine Sulphate.

Culture media
1. MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0 mg l^{-1} adenine sulphate
2. MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 20 mg l^{-1} adenine NAA + 20 mg l^{-1} adenine sulphate
3. MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 40 mg l^{-1} adenine NAA + 20 mg l^{-1} adenine sulphate
4. MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 60 mg l^{-1} adenine sulphate

Addition of D-biotin for Cotyledon Explant Cultures

This experiment was carried out to study the effect of D-biotin on cultured cotyledon explants. The surface sterilized cotyledons were dissected into four quarters and inoculated on 10 ml MS medium supplemented with BAP and NAA alone or in combination with D- biotin as shown in Table 2. All the cultures were maintained under *in vitro* culture conditions after sealing and labelling.

Table 2. Different MS media combined with D-biotin.

Culture media
1. MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0 mg l^{-1} D-biotin
2. MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 1.0 mg l^{-1} D-biotin
3. MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0 mg l^{-1} D-biotin
4. MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 1.0 mg l^{-1} D-biotin

In vitro responses were recorded at regular intervals. Number of days for callus induction, callus formation % and weight of callus were recorded after six weeks of culture. There were

three replicates for each treatment and each replicate contained twelve explants. After six weeks of culture, subculturing was done on MS medium containing 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA for the shoot regeneration.

In Vitro shoot multiplication and Rooting

Microshoots (30 days old) regenerated from the initial cultures were collected from the cultured explants and transferred to the culture vessels containing 10 ml of MS medium with 3.0 mg l^{-1} BAP for shoot multiplication. Subsequently, the cultures were placed under *in vitro* culture conditions. The regenerated shoots (3 - 5 cm long) were then collected from the culture vials and transferred to MS medium supplemented with 1.0 mg l^{-1} IBA for rooting. All cultures were maintained under *in vitro* culture conditions.

Acclimatization

Plantlets were collected from the culture vials and washed to remove adhering agar. Then they were planted in plastic pots containing a mixture of sterilized top soil and sand in the ratio of 1:1. The plantlets were then hardened by keeping the covered plantlets with a polyethylene bag to maintain high relative humidity. A few small holes were made in the polyethylene bags. After 3 weeks, the polyethylene bags were gradually removed and the pots containing plants were then gradually exposed to the natural conditions.

Statistical Analysis

Data of three independent experiments were subjected to analysis of variance (ANOVA) using SAS 9.1.3 portable version. The significant difference between treatment means was estimated using Tukey's Test at 5% significant level.

RESULTS AND DISCUSSION

The suitable explants are one of the key factors for successful plant propagation of bitter gourd through somatic embryogenesis or organogenesis. Plant growth regulators regulate the *in vitro* plant regeneration from the explants cultured on culture media. Further, additives incorporated into the culture media improve the potential of *in vitro* plant regeneration. The present study was therefore carried out to select the most suitable explant for *in vitro* shoot regeneration

and to find out the effect of adenine sulphate and D-biotin on the cultured cotyledon explants. The results of the studies are discussed below.

Selection of Explants for Shoot Induction

The sterilized decoated seeds cultured on MS basal medium exhibited a higher germination percentage (89%) under *in vitro* conditions. It may be due to the removal of the seed coat. The different explants viz, hypocotyl, cotyledon, leaf and cotyledon node excised from *in vitro* seedlings were cultured on MS medium supplemented with 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA to select suitable explant for best morphogenic response. The results revealed that callus was initiated in all types of the cultured explants however it was first induced in the cotyledon explants after two weeks of culture. The callus developed from cotyledon and leaf explants was friable, white in colour while cotyledon node and hypocotyl explants produced slightly compacted callus (Figure 2).

Adventitious roots were developed from the cotyledon callus after four weeks of culture (Figure 2e). Shoot buds were emerged from the cotyledon node after five weeks (Figure 2f) which was from the existing buds in the nodal portion. A remarkable difference (p = 0.0001) was observed in mean days taken for callus initiation among the cultured explants. A minimum day (11.3) was taken to produce callus from the cotyledon explants (Table 3). Callus formation % and mean weight of the callus developed from each explant were recorded after six weeks of culture. There were significant variations in callus formation % (p = 0.0004) and callus weight (p = 0.0002) among the cultured explants. The cotyledon explants showed considerably highest callus weight (0.657 g) than the other explants.

It was noted that cotyledon was the best for callus formation among the different explants tested in the MS medium with 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. The young cotyledons are physiologically active and are influenced by exogenous plant growth regulators (Murkute *et al.,* 2002; Naik and Chand, 2003). Ozyigit *et al.* (2002) reported that cotyledons had a high potential for plant regeneration among the different explants of *Helianthus*. Based on the present findings, cotyledon was most responsive explants for initial culture

establishment under in vitro conditions.

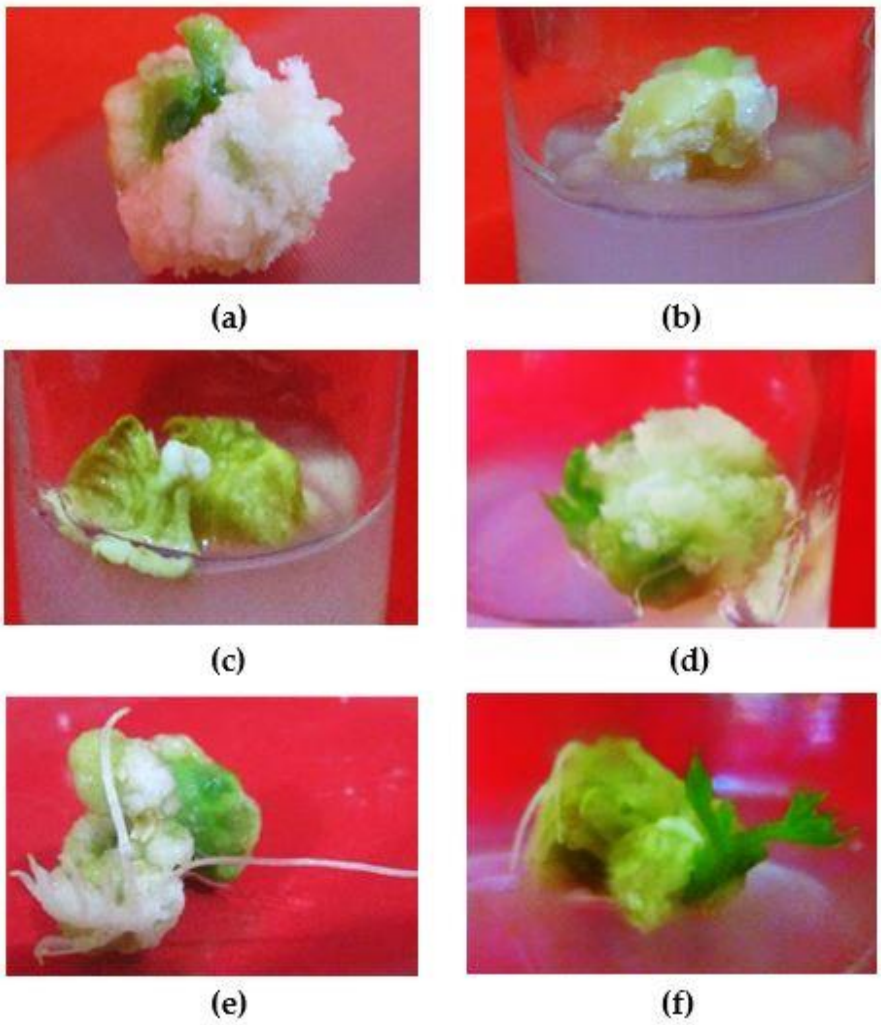

Figure 2. In vitro response of different explants cultured on MS medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA after six weeks of culture. (a) Callus formation from cotyledon, (b) Callus formation from hypocotyls, (c) Callus formation from leaf, (d) Callus formation from cotyledon node explants, (e) Adventitious roots developed from cotyledon explant, (f) Shoot bud emergence from cotyledon node explant.

Table 3. Response of different explants cultured on MS medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA.

Explants	Mean days taken for callus initiation	After six weeks of culture	
		Callus formation (%)	Mean weight of callus (g)
Cotyledon	11.3 ± 0.7c	97.2 ± 2.8a	0.657 ± 0.038a
Cotyledon node	16.7 ± 0.7b	91.7 ± 4.8a	0.462 ± 0.025b
Leaf	17.3 ± 0.8b	91.6 ± 4.8a	0.312 ± 0.016c
Hypocotyl	24.7 ± 0.9a	56.7 ± 3.3b	0.455 ± 0.029b
p value	0.0001	0.0004	0.0002

Values represent means ± standard error of three replicates. Means followed by the same letter in each column are not significantly different based on Tukey's test at 5% significant level.

Effect of Adenine Sulphate on Cotyledon Explants

In this experiment, different concentrations of adenine sulphate (AS) were tested for callus formation and shoot regeneration from cotyledon explants of bitter gourd. It was observed that callus was initiated in all AS concentrations within two weeks but the callus initiation was visible in the cotyledon explants within one week of culture. Further, results showed the rapid callus growth in the cultured cotyledon explants which increased to 2 - 3 folds within four weeks. There was significant variation (p = 0.0039) in time taken for callus initiation from the cotyledon explants on MS medium supplemented with 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and different concentrations of AS. Number of days for callus initiation reduced with increasing concentrations of AS from 0 to 60 mg l^{-1} (Table 4). Thus, minimum days (5.0) was taken for callus induction in the cotyledon explants cultured on MS medium supplemented with highest AS concentration meanwhile the cotyledon explants cultured on the MS medium without AS (control treatment) took maximum days (11.0) to initiate callus.

Table 4. Effect of different concentrations of adenine sulphate on cultured cotyledon explants.

Media with adenine sulphate	Mean days taken for callus induction	After six weeks of culture	
		Callus formation (%)	Mean weight of callus (g)
MS_1 (control)	11.0 ± 1.1a	86.1 ± 2.8	0.43 ± 0.14d
MS_2	8.3 ± 0.9ab	83.3 ± 4.8	1.03 ± 0.10c
MS_3	5.7 ± 0.7bc	91.7 ± 4.8	1.48 ± 0.01b
MS_4	5.0 ± 0.6c	88.9 ± 0.2	2.11 ± 0.13a
p value	0.0039	0.5121	0.0001

MS_1 : MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0 mg l^{-1} adenine sulphate, MS_2 :MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 20 mg l^{-1} adenine sulphate, MS_3 : MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 40 mg l^{-1} adenine sulphate, MS_4 : MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 60 mg l^{-1} adenine sulphate. Values represent means ± standard error of three replicates. Means followed by the same letter in each column are not significantly different based on Tukey's test at 5% significant level.

There was no considerable difference (p = 0.5121) among the different AS concentrations in percentage of callus formation from cotyledon explants. Callus was formed on media with different AS concentrations. It may be due to the addition of BAP and NAA to the culture media which induced callus. BAP and NAA are available in all four media at constant rate. It may be favourable for the callus growth. This is supported by Al Munsur *et al.* (2007) who reported that about 12 days were taken to induce callus from leaf explant cultured on MS medium containing BAP and NAA. In the present study, the peripheral calli expanded whole surface of the cotyledons within four weeks of culture (Figure 3).

On the other hand, remarkable variation (p = 0.0001) was observed in mean weight of callus on the media supplemented with different AS concentrations after six weeks of culture. Considerably low weight (0.43 g) of callus was attained in the medium without AS. Whereas callus weight was remarkably high (2.11 g) on medium containing 60 mgl^{-1} adenine sulphate followed by 40 mg l^{-1} and 20 mgl^{-1} (Table 4). BAP and NAA concentrations were constant for all treatments while AS concentration was increased consistently. Hence, addition of AS may enhance the callus growth

with combination of BAP and NAA. George *et al.* (2008a) mentioned that AS stimulates callogenesis and enhances adventitious shoot formation indirectly from calli or directly from explants when it is coupled with ammonium nitrate or with cytokinins such as BAP. After six weeks of culture, callus was subcultured on MS medium supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA for shoot regeneration thereafter callus morphological appearance was progressively changed. Callus became more nodular and small protuberances were noticeable on the top of these nodular calli (Figure 3). Response for shoot regeneration was positively noted on calli which were cultured on MS medium having 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. After three weeks of subculturing, shoot regeneration was better in callus formed on medium with 60 mg l^{-1} AS, followed by calli obtained from 40 mg l^{-1} AS. Sujata (2013) also stated that addition of adenine sulphate (40 mg l^{-1}) boosted the growth of the shoots with dark green leaves of salvadora plant. Seran *et al.* (2007) reported that typical somatic embryos were produced in immature cotyledon of MS medium supplemented with 2 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. Kamshananthi and Seran (2012) stated that somatic embryos could be obtained from cotyledon explants cultured in MS medium containing BAP alone or in combination with NAA.

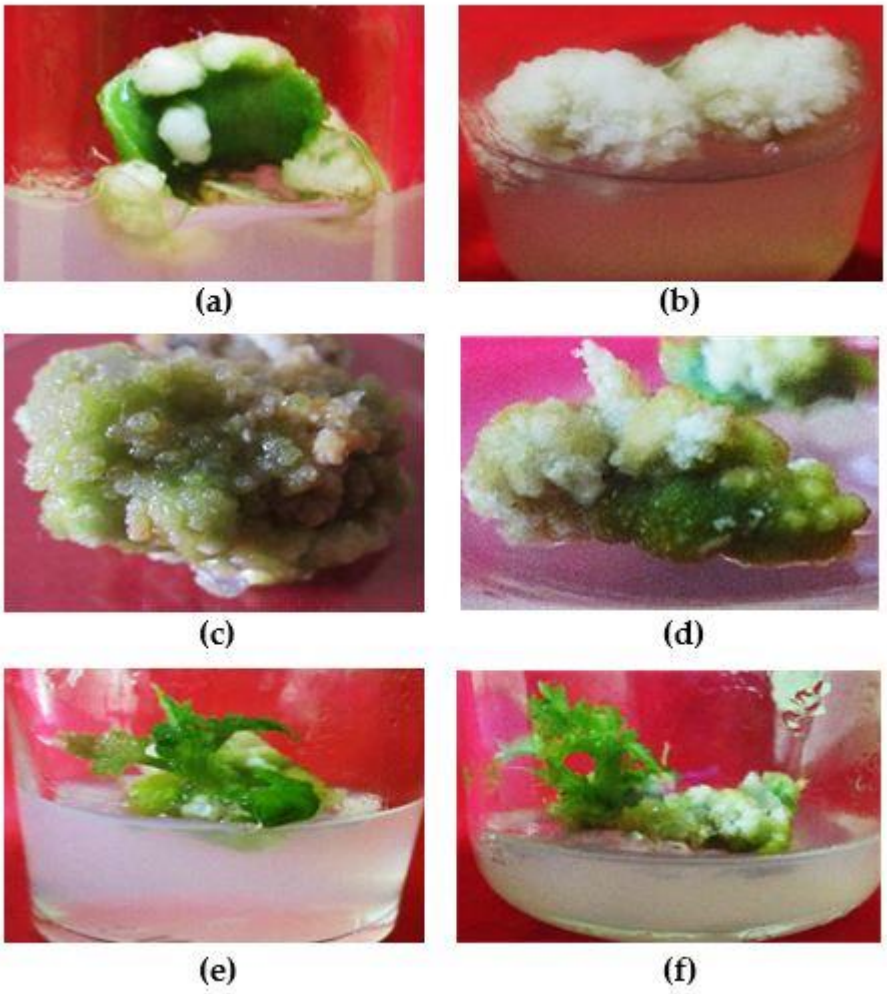

Figure 3. Effect of addition of AS for *in vitro* response of cotyledon explants. (a) Callus growth at 1st week, (b) Callus growth at 3rd week on MS medium containing 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 60 mg l^{-1} AS, (c) Nodular callus sub cultured on MS medium containing 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA, (d) Protuberances development on the top of growing callus, (e) Shoot regeneration from Callus on 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 40 mg l^{-1} AS, (f) Shoot regeneration from Callus on 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 60 mg l^{-1} AS.

The shoots were first appeared as nodular growth within two weeks of subculture on MS media having 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA subsequently nodular structures developed into shoots. Nandagopal and Kumari (2006) reported that regeneration of *in vitro* shoot buds of *Cichoriumi* plant were initiated in thirty days old callus in the presence of AS. In the present study, AS was used in MS medium along with cytokinin

which may enhance the effect of cytokinin and support to shoot regeneration because AS acts as a precursor for natural cytokinin synthesis (Khan *et al*., 2014). This is proved by Imran *et al.* (2012) who noted that a large number of shoots from shoot apex explant of *C. carandas* on medium containing cytokinin along with AS. Further, this is strengthened by Gatica *et al.* (2010) who stated that kinetin in combination with AS is known to enhance organogenesis. AS is useful in the induction of multiple shoots in *Chlorophytum arundinacearum* (Samantaray and Maiti, 2011) and it has proved to be the best for shoot differentiation by Hussain *et al.* (2009) in *Melia azedarach* L. and Begum *et al.* (2007) in *Ophiorrhiza*. The results of this experiment indicated that addition of AS into a nutrient medium is favourable for promoting callus growth and shoot proliferation in cotyledon explant of bitter gourd.

Effect of D-biotin on Cotyledon Explants

The cotyledon explants of bitter gourd were cultured on MS medium supplemented with growth regulators (BAP and NAA) alone and in combination with D-biotin to study the effects of D-biotin on callogenesis. The result showed that callus was initiated at the cut ends of the cultured cotyledon explants. When callus was induced the cotyledons twisted and enlarged. The entire surface of the explants was covered up with the callus within six weeks of culture in all treatments (Figure 4). Mean days taken to callus induction were noted regularly. According to statistical analysis, there was a noticeable difference (p = 0.0026) in mean days taken for callus initiation from the cotyledon in different culture media (Table 5). Minimum days (7.6) for the callus initiation were recorded from the cotyledons on MS medium with 2.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1.0 mg l^{-1} D-biotin (MD4) whereas it was maximum (12.3) on medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA without D-biotin (MD1-control treatment).

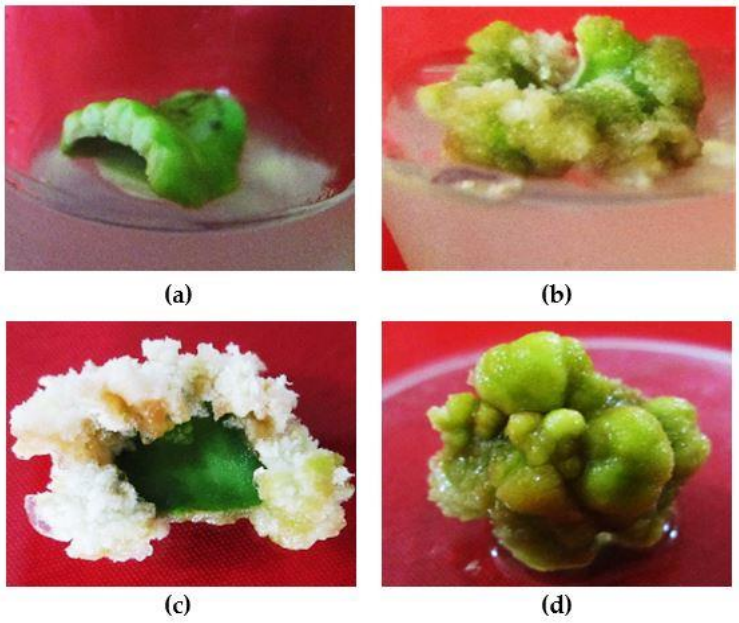

Figure 4. Callus formation from cotyledon explants on MS medium supplemented with 2.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1.0 mg l^{-1} D-biotin. Callus formation on MS medium supplemented with 2.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1.0 mg l^{-1} D- biotin (a) after one week of culture, (b) after two weeks, (c) after three weeks, (d) Callus subcultured on MS medium with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA after 2 weeks.

Table 5. Effect of different concentrations of D-biotin on the cultured cotyledon explants.

Media without or with D-biotin	Mean days taken for callus induction	After six weeks of culture	
		Callus formation (%)	Mean weight of callus (g)
MD_1 (control)	12.3 ± 0.3a	77.8 ± 2.8c	0.516 ± 0.12c
MD2	10.0 ± 0.9b	88.9 ± 2.8ab	1.520 ± 0.09b
MD3	10.0 ± 0.1b	97.2 ± 2.7a	1.493 ± 0.01b
MD4	07.6 ± 0.3c	86.2 ± 2.6bc	1.856 ± 0.06a
p value	0.0026	0.0076	0.0001

MD1: MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0 mgl^{-1} D-biotin. MD2: MS + 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 1.0 mgl^{-1} D-biotin. MD3 MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0 mgl^{-1} D-biotin. MD4: MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 1.0 mgl^{-1} D-biotin. Values represent means ± standard error of three replicates. Means followed by the same letter in each column are not significantly different based on Tukey's test at 5% significant level.

El-Shiaty *et al.* (2004) stated that the exogenous vitamins such as thiamine and biotin are vital for callus induction. In view of the fact, thiamine was already added in basal MS medium, D-biotin accelerated the callus initiation in the present study. Callus formation % and mean weight of callus were recorded after six weeks of culture. It was noted that there was a significant difference among the treatments in the percentage of callus formation and mean weight of callus after six weeks **(**Table 5). The cotyledons on the MS medium with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA (MD3) produced the highest frequency of callus (97.2%) than that with MD4 medium. The lowest callus formation % was (77.8%) was recorded in the control treatment (MD1). Even though the highest percentage of callus formation was achieved in the MD3 medium, the maximum mean weight (1.856 g) was attained in the MD4 medium. Callus weight was lowest from the cotyledons on MD1 medium as compared to that from the other media combinations. This is agreed by Al-Khayri (2001) who mentioned that there is a direct association between biotin concentration and callus weight in date palm and addition of 1.0 mg l^{-1} biotin considerably stimulated callus proliferation.

It was obvious that there was no significant difference between weights of callus formed from MD2 (1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA with 1.0 mg l^{-1} D-biotin) and MD3 (2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA without D-biotin). The result exhibited that low concentration of BAP with D-biotin increase the callus growth. Highest weight of callus was recorded in the medium with 2.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1.0 mg l^{-1} D-biotin. This is in agreement with Abrahamian and Kantharajah (2011) who stated that D-biotin is a vitamin, in combination with other medium components has direct and indirect effects on callus growth. Hence, D-biotin added to the culture medium with BAP and NAA has a significant effect on the callus induction and growth. Callus developed from all four media were subcultured on MS medium supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA after six weeks. Subsequently, nodular structures were observed on the cultured callus but shoots were not regenerated from callus within eight weeks of culture.

Shoot Multiplication

Thirty days old shoots regenerated from selected initial cultures were excised from the original explant and placed on MS medium containing 3.0 mg l^{-1} BAP for shoot proliferation. BAP is used as growth regulator in the culture medium which produced shoot buds after four weeks of culture (Figure 5). This is harmonious with the report of Lakshmi and Mythil (2003) that BAP generally increases shoot multiplication of several medicinal plant species. According to the previous works, Bologun *et al.* (2007) reported that culture medium with 1.5 mg l^{-1} BAP gave good result for shoot of pumpkin while Li *et al.* (2008) stated that for *Cucumis sativus*, shoot proliferation was better in 2.0 mg l^{-1} BAP. Further, BAP alone had a beneficial effective for *in vitro* shoot multiplication in *Momordica charantia* by Agarwal and Kamal (2004) and *Cucumis melo* by Chan and Lok (2005).

In Vitro Rooting

Regenerated shoots having 3-5 cm long were placed on MS medium containing 1.0 mg l^{-1} IBA for rooting of microshoots. After three weeks of culture, root formation was noted in the cultured shoots (Figure 5). This is supported by Kausar *et al.* (2013) and *Debnath et al.* (2013) confirmed that efficient rooting was attained from regenerated shoots cultured on half-strength MS medium containing 1.0 mg l^{-1} IBA. Taware *et al.* (2010) reported that rooting of the *in vitro* derived shoots was optimal when cultured on MS medium with 2.0 mg l^{-1} IBA. George *et al.* (2008b) stated that IBA is effective to increase endogenous auxin contents. In the present study, the rooted plantlets were removed from the culture medium and washed thoroughly and subsequently, they were placed in plastic pots containing a mixture of sterilized topsoil and sand at the ratio of 1:1 for acclimatization.

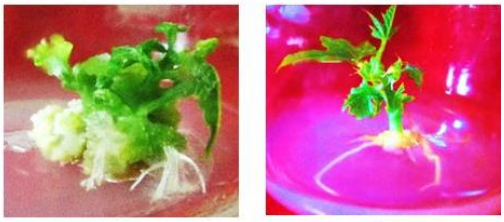

Figure 5. Shoot multiplication and *in vitro* rooting of microshoot. (a) Shoot multiplication on MS medium containing 3.0 mg l^{-1} BAP, (b) *In vitro* rooting on MS medium containing 1.0 mg l^{-1} IBA.

CONCLUSION

Among four types of explants (cotyledon, cotyledon node, leaf, and hypocotyl), cotyledon was the most suitable explant for initial *in vitro* culture establishment which induced callus within minimum period and showed a high percentage (97.2%) of callus formation on MS medium containing 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. It indicated that the type of explant is crucial in callus induction and plant regeneration. Further, callus growth and shoot regeneration was achieved best from cotyledon explants on MS medium containing with 1.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 60 mg l^{-1} adenine sulphate which showed quick response (5 days) of callus and produced the highest callus weight (2.11 g). Callus derived from this medium gave a positive response for shoot regeneration three weeks after subculturing on MS medium with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. This result exhibited that, adenine sulphate incorporation into the culture medium had a beneficial effect on the cotyledon explants of bitter gourd, in association with BAP and NAA. The weight of callus produced in the MS medium supplemented with 2.0 mg l^{-1} BAP, 0.2 mg l^{-1} NAA and 1.0 mg l^{-1} D- biotin was not significantly different from the callus obtained in 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA without D-biotin. Regenerated shoots were successfully multiplied on MS medium containing 3.0 mg l^{-1} BAP. Eventually, *in vitro* rooting was achieved from MS medium with 1.0 mg l^{-1} IBA.

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