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EFFECT OF SUCROSE, BENZYLAMINOPURINE AND CULTURE CONDITION ON *IN VITRO* PROPAGATION OF *CURCUMA XANTHORRHIZA* ROXB. AND *ZINGIBER AROMATICUM* VAL.

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Abstract

Curcuma xanthorrhiza and *Zingiber aromaticum*, are important medicinal species of the Zingiberaceae family. They are used in traditional medicine known as Jamu. This study reports on the presence of sucrose and BA (benzylaminopurine) supplements in the culture medium and also the effect of varying culture condition on *In vitro* propagation of these two species. Murashige & Skoog (MS) medium supplemented with 5 mg L^{-1} BA and 30 g L^{-1} sucrose, incubated under total darkness, induced the highest number of multiple shoots in C. *xanthorrhiza* while the *Z. aromaticum* produced the highest number of multiple shoot in the same medium incubated under continuous light. The increase in sucrose concentration reduced shoot height in both species. Rooting of micro-shoots was not affected by the different culture treatments. The acclimatized plantlets of *C. xanthorrhiza* and *Z. aromaticum* were normal with 83.3-100% percentage when transferred to the outside environment.

Introduction

Zingiberaceae family represents 21 genera and approximately 330 to 350 species in East and West Malaysia (Ibrahim, 1991). Cu*rcuma xanthorrhiza* Roxb., and *Zingiber aromaticum* Val., are important species of this family and are used in traditional medicine known as Jamu. *C. xanthorrhiza*, commonly known as Javanese turmeric, is distributed in India, Indonesia, Thiland and Malaysia (Sirirugsa, 1997). It is used traditionally for the treatment of constipation; stomachache, liver disorders, diarrhea, dysentery, fevers, haemorrhoids and skin eruptions in South-East Asian countries (Lin *et al.,* 1996). The rhizomes of *C. xanthorrhiza* are aromatic and used in the treatment of indigestion, hepatitis, jaundice, diabetes, atherosclerosis and bacterial infections (Kuhn & Winston, 2001; Van Wyk & Wink, 2004). *C. xanthorrhiza* possess hypotriglyceridaemic (Yamazaki *et al.,* 1988), antitumor (Itokawa *et al.,* 1985), anti-inflammatory (Ozaki, 1990), hepatoprotective (Lin *et al.,* 1996) anti-bacterial, anti-inflammatory, anti-asthma (Itokawa *et al.,* 2000) and anticariogenic activities (Hwang *et al.,* 2000). The different chemical constituents such as xanthorrhizol, arturmenone, α-curcumene, β-curcumene, curzerenone, germacrone, β-sesquiphellandrene, α-turmerone and β-turmerone have been isolated from *C. xanthorrhiza* (Uehara *et al.,* 1992). It is reported that *C. xanthorrhiza* contains α-curcumen, ar-turmerone and xanthorrhizol which has strong anti-cancer activities against sarcoma 180 ascites in mice (Kim *et al.,* 1998; Syu *et al.,* 1998; Deters *et al*., 1999). However, xanthorrhizol exhibited antibacterial activity (Chun *et al.,* 1999).

Z. aromaticum, commonly known as Puyang in Malaysia or Lempuyang wangi in Indonesia, contains zerumbone, a monocyclic sesquiterpene in its rhizome (Murakami *et al.,* 2002). Zerumbone has a great potential for use in chemoprevention and chemotherapy strategies against cancers (Lee, 1993; Takada *et al.,* 2005). Zerumbone also found to exhibite HIV-inhibitory and cytotoxic activities (Dai *et al.,* 1997).

Generally, C. *xanthorrhiza* and *Z. aromaticum* are propagated vegetatively via rhizomes. However, large quantities of rhizomes are often required because the efficiency of vegetative propagation is low. Therefore, plant tissue culture techniques have been found to be useful in the conservation, mass propagation of plantlets and production of bioactive compounds and for genetic improvement (Sivanesan *et al.,* 2012; Abbas & Qaiser, 2012; Hui *et al.,* 2012; Bhatt *et al.*, 2013). Uniform plant materials can be obtained without the influence of environmental factors via the tissue culture technique. Moreover, it offers a sustainable supply of biochemicals independent of plant availability and more consistent product quality (Sajc *et al.,* 2000; Garshasbi *et al*., 2012).

In vitro propagation protocols have been established for some Zingiberaceae species such as *Alpinia galangal* (Salvi *et al.,* 2002), *Curcuma haritha* (Bejoy *et al.,* 2006), *Curcuma zedoaria* and *Zingiber zerumbret* (Stanly & Chan, 2007; Stanly & Chan, 2007; Stanly *et al.,* 2010; Stanly *et al.,* 2011). Micropropagation of *Curcuma xanthorrhiza* using rhizome as explants has been reported by Lu *et al.,* (2007). However no information is available on the combination effect of sucrose, benzylaminopurine (BA) and different culture condition (continuous light and total darkness) on *In vitro* propagation of C. *xanthorrhiza* and *Z. aromaticum*. Therefore, the present study investigates the effect of different concentration of sucrose, BA and culture condition on plantlets production of C. *xanthorrhiza* and *Z. aromaticum*.

Material and Methods

Effect of BA, sucrose and photoperiod on shoot proliferation: *In vitro* shoots (3.0 cm) of C. *xanthorrhiza* and *Z. aromaticum,* established from the rhizome buds, were used as study materials. The shoot explants consisted of leave sheaths with one apical shoot per explant. They were inoculated into glass test tubes [15 cm (h) x 2.5 cm (diameter)] containing Murashige & Skoog (1962) medium (MS) supplemented with different

concentrations of BA (0, 1, 3 and 5mgL^{-1}) and sucrose (30, 60 and 90gL^{-1}). Cultures were placed under varying photoperiod i.e., continuous light with light intensity 32.5 µE m^{-2} s^{-1} and total darkness. The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving at 121°C for 11 minutes. All the cultures were maintained at 25 ± 2ºC in a culture room. The experiment was conducted in 4 x 3 x 2 factorial design with three replicates for each treatment. The experiment was repeated twice. The number of shoots produced from each explants, shoot length, number of leaves, number of roots and the roots' lengths were determined after 8 weeks of culture. The data were analyzed using 3-way analysis of variance (ANOVA) in order to test the main effect of BA, sucrose and photoperiod as well as the interaction among these factors and their combined effect on the growth characteristic of C. *xanthorrhiza* and *Z. aromaticum* plantlets. The comparison of means was carried out using Tukey Test at $p \leq 0.05$ with the aid of SPSS version 12 (SPSS Inc., Chicago, Illinois) to test the significance.

Acclimatization: Eight weeks old rooted *In vitro* plantlets of both the species obtained from MS medium supplemented with 1gL^{-1} BA and 30gL^{-1} sucrose under different incubation were removed from the culture vessels and washed thoroughly under running tap water. The plantlets were than transplanted into plastic tray (30 x 40cm) containing a mixture of organic soil and sand (1:1). These trays were placed in a green house and the plantlets were exposed to a relative humidity of 80-90% and temperature of 28 ± 2ºC during day time and 24 ± 2ºC during night time. The plantlets were watered twice a day (morning and evening) with tap water. The percentage of surviving plantlets, shoot height and number of leaves formed were recorded after 6 weeks of acclimatization. Shoot height was measured from the soil level to the tip of the youngest emerging leaf.

Effect of *In vitro* culture conditions on the anatomical features of the leaf and root: The leaves and roots of eight week old *In vitro* plantlets of *C. xanthorrhiza* and *Z. aromaticum* incubated under continuous light and total darkness was randomly selected and cut into small pieces and fixed in FAA (formalin:acetic acid:ethanol 1:1:18). The fixed samples were washed with distilled water for five times. After washing, the specimens were dehydrated by passing through a tertiary butyl alcohol– xylol series and embedded in paraffin wax (Johansen 1940). The specimens were cut into blocks and serial sections (10–12 µm) were made using a rotary microtome (Olympus 4060 microtome). The sections were mounted onto slides and allowed to dry for 24 h before staining. The specimens were stained with safranin and fast green. Cover slips were mounted with histoclad mounting medium and dried. The permanent slides were observed under a microscope fitted with a camera (Olympus Bx50).

Results and Discussion

Effect of BA, sucrose and photoperiod on shoot proliferation

***C. xanthorrhiza*:** The presence of different sucrose concentration (30, 60 and 90 g L^{-1}), BA (1, 3 and 5 mg L^{-1}) and incubation under different photoperiods (continuous light and total darkness) were found to affect the shoot proliferation of *C. xanthorrhiza*. MS medium supplemented with different sucrose concentrations (without BA), induce 1.7-3.2 shoots/explant under continuous light and 1.8-2.3 shoots/explant under total darkness after eight weeks of culture (Table 1).

MS medium supplemented with 5 mg L^{-1} BA and 30 g L^{-1} sucrose incubated under total darkness induced the highest number of multiple shoots (5.2 shoots/explants), while the results were significantly different when the culture medium was supplemented with 1 and 3 mg L^{-1} BA and 90 g L^{-1} sucrose under continuous light and MS medium supplemented with 1, 3 and 5 mg L^{-1} BA and 90 g L^{-1} sucrose and placed under total darkness (Table 1).

Results obtained indicate that the presence of BA with high sucrose content (60gL^{-1} and 90gL^{-1}) suppresses the *In vitro* shoot height of *C. xanthorrhiza*. However, MS medium supplemented with 30gL^{-1} of sucrose (without BA) show maximum shoot height (5.3 cm) under total darkness which is significantly higher than the most of the applied treatments except MS medium supplemented with 30gL^{-1} (without BA), and MS plus 1, 3 and 5 mg L^{-1} BA supplemented with 30 g L^{-1} sucrose under continuous light or total darkness. *C. xanthorrhiza* showed the maximum increment in shoot height in MS medium supplemented with 30gL^{-1} of sucrose (without BA) (Table 1).

The highest number of leaves produced for each shoot explant (9.3 ± 4.7) for *C. xanthorrhiza* was obtained in plantlets cultured on MS medium supplemented with 5mgL^{-1} BA and 30gL^{-1} sucrose under continuous light. This was significantly higher than all the treatments incubated under total darkness. However, there was no significant difference in terms of leaves production between MS medium supplemented with 5mgL^{-1} BA and 30gL^{-1} sucrose incubated under continuous light and MS medium supplemented with 1 and 3mgL^{-1} BA and 30g L^{-1} sucrose and MS plus 3 and 5mg L^{-1} BA and 60g L^{-1} sucrose under continuous light (Table 1).

All the applied treatments were found to be effective for root induction in *C. xanthorrhiza*. MS medium supplemented with 5mg L^{-1} BA and 60g L^{-1} sucrose under continuous light induced the highest number of roots (22.0 root/shoot explant) which is significantly higher than the most of applied treatments except MS medium supplemented with 3mgL^{-1} BA and 60gL^{-1} sucrose and 5mgL^{-1} BA and 30gL^{-1} sucrose under continuous light. Higher sucrose content (90gL^{-1}) in the culture medium reduced the number of roots in *C. xanthorrhiza* under contentious light and total darkness (Table 1).

The maximum root length was achieved in the MS medium supplemented with 1 and 3mgL^{-1} BA and 30gL^{-1} sucrose under continuous light and MS medium supplemented 30gL^{-1} sucrose (without BA) under total darkness. This was significantly higher than the length of root produced in MS medium supplemented 90gL^{-1} sucrose (without BA), 3 and 5mgL^{-1} BA and 90gL^{-1} sucrose under continuous light and 1, 3 and 5mgL^{-1} BA and 90gL^{-1} sucrose under total darkness in C. *xanthorrhiza* (Table 1).

Table 1. Effect of sucrose, BA and photoperiod on *In vitro* growth of *C. xanthorrhiza* plantlets.

Treatment variables			Parameters				
Continuous light/ total darkness	**BA (mg l^{-1})**	**Sucrose concentration (g l^{-1})**	**Shoot height (cm ± SE)**	**Number of multiple shoot (n ± se)**	**Number of root (n ± se)**	**Root length (cm ± se)**	**Number of leaves (n+se)**
Light	0	30	4.4 ± 1.1 ghi	1.8 ± 0.4 abc	7.7 ± 2.7 abcde	1.6 ± 0.5 de	4.2 ± 1.5 abcdef
Light	0	60	3.1 ± 0.6 bcdefgh	3.2 ± 1.7 abcde	7.8 ± 5.0 abcde	1.4 ± 0.3 abcde	4.7 ± 2.6 cdef
Light	0	90	1.6 ± 1.0 abc	1.7 ± 1.0 ab	5.7 ± 1.9 abc	0.6 ± 0.3 ab	1.8 ± 1.0 abc
Light	1	30	3.4 ± 1.3 bcdefghi	3.7 ± 1.2 abcde	15.5 ± 6.7 efgh	1.7 ± 0.5 e	7.2 ± 4.8 fg
Light	1	60	1.8 ± 0.4 abcd	4.2 ± 1.2 abcde	13.7 ± 5.5 defg	0.9 ± 0.1 abcde	4.3 ± 0.8 abcdef
Light	1	90	0.8 ± 0.6 a	1.5 ± 0.9 a	3.8 ± 1.5 a	1.1 ± 0.5 abcde	1.2 ± 0.6 abc
Light	3	30	3.5 ± 1.0 cdefghi	2.8 ± 1.2 abcde	12.8 ± 3.3 cdefg	1.7 ± 0.7	5.8 ± 1.7 defg
Light	3	60	2.7 ± 0.9 abcdefgh	4.0 ± 1.1 abcde	17.7 ± 5.0 fgh	1.6 ± 0.5 de	5.8 ± 1.8 defg
Light	3	90	1.8 ± 1.3 abcd	2.2 ± 1.7 abcd	3.8 ± 2.4 a	0.7 ± 0.6 abc	1.8 ± 0.9 abc
Light	5	30	3.7 ± 0.8 defghi	4.2 ± 1.2 abcde	20.7 ± 7.7 gh	1.6 ± 0.5 de	9.3 ± 4.7 g
Light	5	60	2.5 ± 0.6 abcdefg	4.7 ± 0.8 de	22.0 ± 6.9 h	1.2 ± 0.4 abcde	6.8 ± 1.5 efg
Light	5	90	1.5 ± 0.3 ab	3.2 ± 1.5 abcde	4.8 ± 3.6 ab	0.6 ± 0.1 abc	2.5 ± 1.1 abcd
Dark	0	30	5.3 ± 1.1 i	1.8 ± 0.4 abc	4.3 ± 2.2 ab	1.7 ± 0.4 e	2.3 ± 0.5 abcd
Dark	0	60	3.1 ± 1.8 bcdefgh	2.5 ± 2.3 abcde	5.7 ± 2.8 abc	1.0 ± 0.6 abcde	1.5 ± 1.1 abc
Dark	0	90	2.7 ± 0.7 abcdefgh	2.3 ± 1.6 abcd	6.0 ± 3.0 abcd	1.0 ± 0.2 abcde	1.7 ± 1.2 abc
Dark	1	30	4.0 ± 1.1 efghi	3.0 ± 0.9 abcde	7.7 ± 1.9 abcde	1.6 ± 0.1 cde	2.5 ± 1.1 abcd
Dark	1	60	2.9 ± 1.0 bcdefgh	4.0 ± 1.3 abcde	9.9 ± 4.3 abcdef	1.3 ± 0.2 abcde	2.3 ± 1.4 abcd
Dark	1	90	2.0 ± 1.2 abcde	1.8 ± 1.2 abc	3.8 ± 1.9 a	0.7 ± 0.5 abc	0.7 ± 0.8 ab
Dark	3	30	4.7 ± 0.5 hi	4.3 ± 0.5 bcde	10.7 ±`3.2 abcdef	1.2 ± 0.2 abcde	5.0 ± 0.6 cdef
Dark	3	60	3.6 ± 1.4 defghi	4.5 ± 0.5 cde	12.0 ± 3.0 bcdef	1.4 ± 0.5 abcde	3.0 ± 1.3 abcde
Dark	3	90	2.3 ± 0.4 abcdef	2.3 ± 1.0 abcd	5.3 ± 1.8 abc	0.8 ± 0.2 abcd	1.5 ± 0.6 abc
Dark	5	30	4.1 ± 1.2 fghi	5.2 ± 2.5 e	9.3 ± 4.1 abcde	1.5 ± 0.4 bcde	4.5 ± 1.8 bcdef
Dark	5	60	2.6 ± 1.0 abcdefg	4.3 ± 1.5 bcde	7.2 ± 3.4 abcd	1.1 ± 0.8 abcde	1.3 ± 1.4 abc
Dark	5	90	1.5 ± 1.0 abc	1.8 ± 1.1 abc	3.0 ± 2.1 a	0.6 ± 0.4 a	0.5 ± 0.3 a

Mean values within the same column followed by the same alphabet are not significantly different (Tukey's HSD test, $p \leq 0.05$)

***Z. aromaticum*:** *Z. aromaticum* produced 0.3- 4.3 shoots/explant in MS medium supplemented with different sucrose concentrations (without BA) incubated under continuous light and 1.5-4.2 shoots/explant under total darkness (Table 2). *Z. aromaticum* also produced highest number of multiple shoot (5.5 shoots/explant) in MS medium supplemented with 5mg L^{-1} BA and 30g L^{-1} sucrose under continuous light. However, the number of multiple shoots produced in this treatment was significantly higher than the number of multiple shoots produced in MS medium supplemented with 90gL^{-1} sucrose without BA or with 1, 3 and 5mg L^{-1} BA under continuous light and total darkness. The addition of higher sucrose concentrations (90 g L-1) seeks to reduce the shoot multiplication rate of *Z. aromaticum* under both continuous light and total darkness (Table 2).

MS medium supplemented with or without different concentrations of BA and sucrose showed an average shoot height between 1.2-3.2 cm after eight weeks of culture under continuous light. While, the cultures using the same medium constituents but incubated under total darkness showed higher shoot height (1.5 to 6.4 cm). MS medium supplemented with 60 g L^{-1} sucrose (without BA) showed the maximum increase in shoot height under total darkness (6.4 cm) which is significantly higher than the most of the applied treatments except MS medium supplemented with 30 g L^{-1} (without BA) and MS medium supplemented with 1 mg L^{-1} BA and 30 g L^{-1} sucrose under total darkness (Table 2).

The highest number of leaves/shoot explant (8.2 ± 1.5) for *Z. aromaticum* was achieved in plantlets cultured on MS medium supplemented 30gL^{-1} sucrose (without BA) under continuous light. They were significantly higher than all the other treatments incubated under total darkness or continuous light except MS medium supplemented with 1 and 5mgL^{-1} BA and 30gL^{-1} sucrose under continuous light (Table 2).

All the tested treatments were also found to have an effect on root induction in *Z. aromaticum*. MS medium supplemented with 1mgL^{-1} BA and 30gL^{-1} sucrose under continuous light induced the highest number of roots (26.7 roots/shoot explant) which is significantly higher than most of the applied treatments except MS medium supplemented 30gL^{-1} sucrose (without BA), MS plus 1 and 5mgL^{-1} BA and 30gL^{-1} sucrose under continuous light. Higher sucrose content (90 g L^{-1}) in the culture medium reduced the number of roots in *Z. aromaticum* under both incubation conditions (Table 2).

The maximum root length (5.0 cm) was obtained in the MS medium supplemented with 30gL^{-1} sucrose (without BA) under continuous light followed by MS medium supplemented with 30 g L^{-1} (4.9 cm) and 60 g L^{-1} (3.9 cm) sucrose (without BA) under continuous dark (Table 2). These treatments induced maximum root length which is significantly higher than the other treatments.

In general, the high sucrose concentration inhibits shoot proliferation of both species when incubated under continuous light and total darkness. Sucrose is considered to be an important source of carbon and energy sources in the culture medium, which also acts as an osmoticum (Hartmann *et al.,* 1997). High sucrose content in the culture medium created high osmotic potential and resulted in slow absorption of nutrients and hence slows down the shoot proliferation rate of both species under studied. Our findings are in agreement with Boufleuher *et al.,* (2008) who reported that MS medium supplemented with 30 g L^{-1} sucrose enhanced shoot regeneration in *Solanum sessiliflorum* as compared to higher sucrose concentration. Similarly, the shoot proliferation rate in *Rosa hybrida* was highest in lower sucrose concentration as compared to higher concentration (Al-Khalifah *et al.,* 2005).

Both the species produced higher number of multiple shoots under higher concentration of BA (5mg/L). Generally, cytokinins (BA) play an important role in cell division and also break bud dormancy, therefore it influences the shoot proliferation rate (Preece, 1995). However, type of cytokinin and their concentration are the major factors that determined the success of *In vitro* multiplication (Grattapaglia *et al.,* 1998). The stimulating effect of BA on shoot proliferation has been reported earlier for several medicinal plant species such as *Bacopa monniera* (Tiwari *et al.,* 2001) *Plumbago zeylanica* (Sivanesan, 2007) *Aloe vera* (Kalimuthu *et al.,* 2010).

Culture condition seem to play an important role in determining the plant growth and shoot proliferation as it is evident in the present study. *C. xanthorrhiza* produced highest number of multiple shoot under total darkness. However, *Z. aromaticum* produced highest number of multiple shoot under continuous light. The different photoperiod requirement of *C. xanthorrhiza* and *Z. aromaticum* may be attributed to the different genetic make up of the species. The present results could be useful in future improvement of the efficiency of micropropagation of *C. xanthorrhiza* and *Z. aromaticum*.

It was reported that the addition of sucrose in the culture medium could increase photosynthesis rate only up to a certain level. Therefore the increase of sucrose concentration in the culture medium beyond that level is likely to reduce photosynthesis rate (Hdider & Desjardins, 1994). In the present study, C. *xanthorrhiza* and *Z. aromaticum* showed the maximum increase in shoot height in MS medium supplemented with 30 and 60 g L^{-1} of sucrose (without BA) respectively. By increasing the sucrose concentration the shoot height will be reduced in both species. Our findings are in tune with Wainwright & Scrace (1989) who reported that by increasing the concentration of sucrose to 20 g L^{-1} level the shoot height in *Potentilla fruticosa* and *Ficus lyrata* will increase However, a further increase of the sucrose concentration beyond that level was found to decrease the shoot height of these species. Addition of BA in the culture medium was found to be in effective when it comes to increase in the shoot height of both species. A similar result was obtained for *Swainsona formosa* where the presence of BA in the culture medium decreased the shoot height (Jusaitis, 1997).

Table 2. Effect of sucrose, BA and photoperiod on *In vitro* growth of *Z. aromaticum* plantlets.

Treatment variables			Parameters				
Continuous light / total darkness	**BA (mg l^{-1})**	**Sucrose concentration (g l^{-1})**	**Shoot height (cm ± SE)**	**Number of multiple shoot (n+se)**	**Number of root (n+se)**	**Root length (cm+se)**	**Number of leaves (n+se)**
Light	0	30	2.9 ± 0.6 abc	4.3 ± 1.4 cde	18.2 ± 4.1 ghi	5.0 ± 1.4 h	8.2 ± 1.5 c
Light	0	60	2.3 ± 1.0 ab	3.8 ± 1.9 bcde	6.3 ± 3.4 abc	1.5 ± 1.1 abcde	0.8 ± 0.8 a
Light	0	90	1.2 ± 0.8 a	0.3 ± 0.1 a	0.6 ± 0.2 a	0.3 ± 0.3 a	0.0 ± 0.0 a
Light	1	30	2.7 ± 0.4 abc	4.3 ± 1.2 cde	26.7 ± 1.6 i	3.0 ± 0.7 fg	7.3 ± 1.5 c
Light	1	60	1.9 ± 1.0 ab	2.5 ± 1.6 abcde	1.8 ± 1.5 a	0.7 ± 0.5 abc	0 a
Light	1	90	3.0 ± 0.3 abc	1.5 ± 0.8 abc	3.2 ± 1.0 ab	0.6 ± 0.1 ab	0 a
Light	3	30	1.8 ± 0.6 ab	5.0 ± 0.9 e	16.8 ± 7.0 efgh	1.8 ± 0.9 bcdef	3.3 ± 2.4 b
Light	3	60	1.7 ± 0.2 ab	4.8 ± 2.1 de	9.0 ± 2.5 abcdef	1.5 ± 0.3 abcde	0.7 ± 0.8 a
Light	3	90	2.0 ± 0.8 ab	1.8 ± 0.4 abcd	2.0 ± 0.6 ab	0.7 ± 0.3 abc	0 a
Light	5	30	1.7 ± 0.4 ab	5.5 ± 2.1 e	20.7 ± 6.5 hi	2.2 ± 0.5 def	6.2 ± 3.4 c
Light	5	60	1.4 ± 0.4 a	4.3 ± 1.6 cde	5.0 ± 2.5 abc	1.0 ± 0.5 abcd	0.2 ± 0.4 a
Light	5	90	3.2 ± 0.2 abc	1.0 ± 0.0 ab	2.0 ± 0.9 ab	0.5 ± 0.1 a	0 a
Dark	0	30	5.4 ± 1.3 de	4.2 ± 1.2 cde	8.5 ± 1.4 abcde	4.9 ± 1.2 h	1.7 ± 0.5 ab
Dark	0	60	6.4 ± 0.5 e	4.0 ± 1.1 bcde	12.8 ± 2.3 cdefgh	3.9 ± 0.8 gh	1.2 ± 1.0 ab
Dark	0	90	3.1 ± 0.9 abc	1.5 ± 0.5 abc	1.2 ± 0.8 a	0.4 ± 0.2 a	0.3 ± 0.5 a
Dark	1	30	4.6 ± 1.7 cde	4.0 ± 2.0 bcde	15.2 ± 7.0 defgh	2.8 ± 0.9 fg	1.3 ± 0.4 ab
Dark	1	60	2.1 ± 0.9 ab	3.8 ± 0.4 bcde	7.7 ± 2.2 abcd	2.3 ± 0.4 ef	0 a
Dark	1	90	2.6 ± 1.3 abc	1.0 ± 0.7 ab	1.0 ± 0.4 a	0.3 ± 0.2 a	0 a
Dark	3	30	3.6 ± 0.9 bcd	4.7 ± 1.6 de	17.5 ± 5.0 fgh	3.1 ± 0.8 fg	2.2 ± 1.3 ab
Dark	3	60	2.7 ± 1.0 abc	4.3 ± 1.5 cde	10.5 ± 6.3 bcdefg	2.8 ± 0.7 efg	0.5 ± 0.8 a
Dark	3	90	1.9 ± 0.7 ab	1.3 ± 0.8 abc	1.5 ± 0.8 a	0.5 ± 0.3 ab	0 a
Dark	5	30	3.0 ± 0.4 abc	3.5 ± 2.2 bcde	13.0 ± 7.2 cdefgh	2.9 ± 1.0 fg	0.8 ± 0.2 a
Dark	5	60	2.1 ± 0.5 ab	3.5 ± 2.1 bcde	8.5 ± 5.7 abcde	2.0 ± 0.6 cdef	0.2 ± 0.1 a
Dark	5	90	1.5 ± 1.2 a	1.0 ± 0.9 ab	0.8 ± 0.4 a	0.4 ± 0.4 a	0.2 ± 0.1 a

Mean values within the same column followed by the same alphabet are not significantly different (Tukey's HSD test, $p \leq 0.05$)

Cost effectiveness and success of any micropropagation protocol depends on the percentage rooting and survival of the plantlets in field conditions (Martin, 2003). All the applied treatments were found effective for root induction in both the species. However, the root production was affected by different concentration of BA and sucrose. MS medium supplemented with 5 mg L^{-1} BA and 60 g L^{-1} and 1 mg L^{-1} BA and 30 g L^{-1} was found to be the best combination for root induction in *C. xanthorrhiza* and in *Z. aromaticum* respectively. Previous study reported that MS medium supplemented with higher concentration of BA reduced the number of roots but the medium without BA could not induce the root formation in banana *In vitro* shoots (Wong, 1986). Presence of higher concentration of BA also inhibited the number of roots production in *Cicer arietinum* (Polisetty *et al.,* 1997). Rooting is energy demanding process requiring an adequate level of carbon source however this may vary from species to species (Hazarika, 2003; Zapata *et al.,* 2003; Asim, 2012). However in the present study both the species responded differently to the different concentration of BA and sucrose indicating that there is a difference in optimal requirements for exogenous supply of sucrose and BA to the culture medium. This may also be attributed to the genotypic difference in between C. *xanthorrhiza* and *Z. aromaticum.* MS medium supplemented with BA 1 mg L^{-1}, 30 g L^{-1} sucrose could be selected as the most suitable proliferation medium for both *C. xanthorrhiza* and *Z. aromaticum.* The shoot proliferation process, in this case, could be carried out either under continuous light or total darkness.

Acclimatization: Rooting of *In vitro* shoots is important and it affects the survival rate of plant in acclimatization process (Goncalves *et al.,* 1998). Acclimatized plantlets of *C. xanthorrhiza* and *Z. aromaticum* did not show any morphological abnormality and survived well (Fig. 1A and B). The plantlets of *C. xanthorrhiza* and *Z. aromaticum* derived from MS medium supplemented with 1 g L^{-1} BA and 30 g L^{-1} sucrose and incubated under either continuous light or total darkness produced high number of roots with generally high survival rates (83.3%-100) (Table 3). Plantlets of *Z. aromaticum* incubated in total darkness, produced fewer leaves with shorter shoot (Table 3). However, plantlets of *C. xanthorrhiza* did not manifest any difference in shoot height and number of leaves produced when grew under two different incubation condition (continuous light and total darkness). This indicates that the different species responds differently under varied light conditions when cultured *In vitro*.

Table 3. Survival percentage and growth characteristics of acclimatized *C. xanthorrhiza* and *Z. aromaticum* plantlets.

Species	Incubation culture	Parameter		Survival %
		Shoot height (cm ± SE)	Number of leaves (n ± SE)	
C. xanthorrhiza	Continuous light	6.3 ± 1.1 a	5.0 ± 1.7 a	100
	Total darkness	6.2 ± 1.4 a	3.7 ± 1.0 a	83.3
Z. aromaticum	Continuous light	10.8 ± 0.7 a	9.8 ± 0.5 a	100
	Total darkness	6.8 ± 0.8 b	3.8 ± 0.3 b	100

Mean values within the same column for each species followed by the same alphabet are not significantly different (Student T-Test test, $p \leq 0.05$)

Effect of *In vitro* culture conditions on anatomical features of the leaf and root: The effect on the anatomical features of leaves and roots of both species incubated under continuous light/ total darkness condition was similar. The upper epidermal layer consists of a single layer of rectangular cells and the chloroplasts are mainly concentrated in the mesophyll tissue of *C. xanthorrhiza* and *Z. aromaticum* (Figs. 2 A & C). The leaf section clearly showed the 2 rows of mesophyll cells in *Z. aromaticum* (Figs. 2 C & D). However, in *C. xanthorrhiza* the arrangement of the mesophyll cells was not distinct (Fig. 2A). Large metaxylem vessels were evident and showed scalariform thickening in the root section of both the species (Fig. 2E and F). These results are in tune with Sherlija *et al.,* (1998) who reported similar anatomical features of the curcuma species. Root shows arcs of xylem alternating with phloem. Stele is well preserved and endodermis and pericycle were clearly visible (Fig. 2E). Some pigmentation could be seen in the cells that could be phonetics (Fig. 2F).

Conclusion

The present investigation identifies some improvement opportunities in *In vitro* shoot multiplication process for *C. xanthorrhiza* and *Z. aromaticum*. The techniques described afore could potentially facilitate production of a large number of uniform plantlets of *C. xanthorrhiza* and *Z. aromaticum* irrespective of season and also could be used as tools for conservation of these species.

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Fig. 1. Acclimatized plantlets of *C. xanthorrhiza* (A) and *Z. aromaticum* (B).

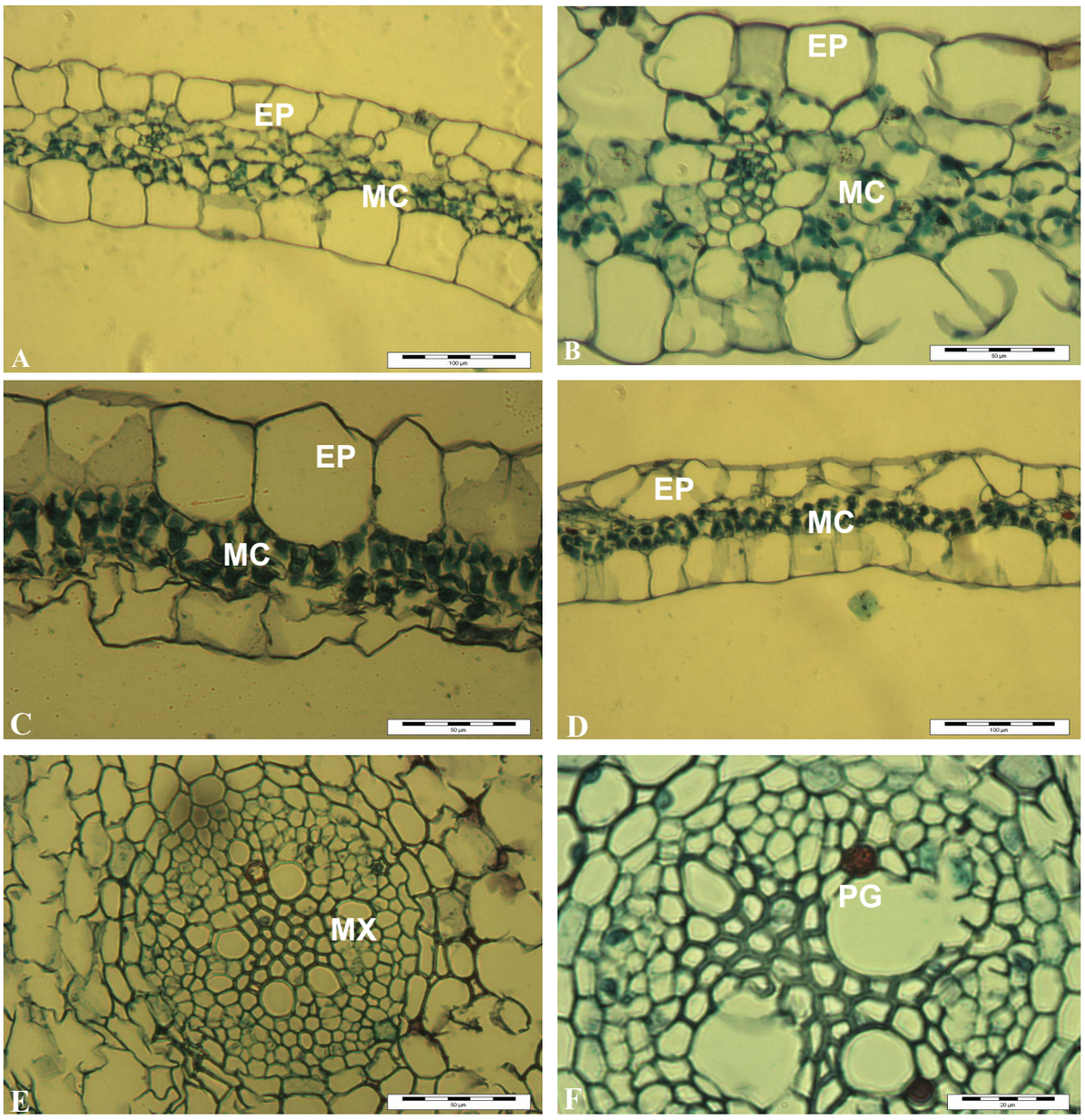

Fig. 2. A-*C. xanthorrhiza* (Light - leaf), B- *C. xanthorrhiza* (Dark - leaf), C-*Z. aromaticum* (light - leaf) D-*Z. aromaticum* Dark - leaf); E-*C. xanthorrhiza* (root); F-*Z. aromaticum (*root) (EP- epidermis; MC- mesophyll cell; MX- metaxylum; PG- pigments).

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