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Micropropagation and antimicrobial activity of *Curcuma aromatica* Salisb., a threatened aromatic medicinal plant

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Abstract: A rapid and improved micropropagation protocol was developed for *Curcuma aromatica*, a threatened aromatic medicinal plant, using rhizome sprout as the explant. Stepwise optimization of different plant growth regulators, carbon sources, and basal media was adopted to establish an efficient micropropagation protocol. When cytokinins, such as benzyl amino purine (BAP) or 6-(α,α-dimethylallylamino)-purine (2iP), were used either singly or in combination with naphthalene acetic acid (NAA) for shoot induction and multiplication, a single use of BAP was the most effective. As a carbon source, 3% (w/v) sucrose exhibits the greatest promotive effect on shoot initiation and proliferation compared with other carbon sources used. Among the basal media, full strength Murashige and Skoog (MS) media produced the best results, compared to other media studied. By using the most effective treatment from each category, an average of 13.2 shoots/per explant were produced after 6 weeks of culture. Moreover, 85% survival was achieved when rooted explants acclimatized ex vitro using a mixture of sterile sand, soil, and farmyard manure (1:1:1). In addition, antimicrobial activities of rhizome extracts were evaluated. Petroleum ether and chloroform extracts of field-grown rhizome showed potential antimicrobial properties against several human pathogenic bacteria including *Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella sonnei,* and *Shigella dysenteriae*, with a minimum inhibitory concentration (MIC) ranging from 0.03 to 0.5 mg/mL. Thus, the optimized micropropagation protocol may offer large-scale production of plantlets to meet industrial demand for the rhizome. Moreover, our results suggest the rhizome extract of *C. aromatica* is a promising antimicrobial agent.

Key words: Antimicrobial activity, *Curcuma aromatica*, endangered, in vitro culture

1. Introduction

Curcuma aromatica Salisb. (Zingiberaceae), indigenous to South Asia, is a robust zingiber with stout underground rhizomes. This wild, aromatic, and attractive turmeric is probably the most useful among the turmeric members for its unique medicinal values. *C. aromatica* rhizome is a rich source of volatile oil, which consists of several major anti-tumor ingredients including demethoxycurcumin, β-elemene, curcumol, curdione, etc. (Zhou et al., 1997; Dulak, 2005). *C. aromatica* could promote blood circulation to remove blood stasis and treat cancers and angiogenesis (Kim et al., 2002). The oil infused via the hepatic artery has proven to exert ideal therapeutic effects in humans with primary liver cancer and rats with transplanted hepatoma (Cheng et al., 1999). Curcumin, the most prevalent active secondary ingredient in *C. aromatica*, acts as a promising agent in the treatment and/or prevention of Alzheimer's disease (Ringman et al., 2005). In addition, the rhizome is

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also used against various bacterial and fungal diseases in traditional medicine. The rhizome extract also possesses a significant repellent activity against mosquitoes (Pitasawat et al., 2003).

Improper cultivation practices, habitat destruction, deforestation, and the greater dependency of pharmaceutical industries on wild sources make this plant threatened in many South Asian countries (Kumar and Sikarwar, 2002). Commercial cultivation of this plant species is also limited by a number of constrains. Conventional propagation by rhizome separation is very slow (Nambiar et al., 1982; Nayak, 2000) and inadequate to meet the demand for plants for commercial cultivation. In addition, rhizomes are highly susceptible to rot disease caused by *Pythium* species in field and storage. Field plants are also susceptible to *Colletotrichum* leaf spot. These are the constraints for the conservation and storage of germplasm in clonal repositories (Nayak, 2000). Uniformity of active ingredients is the most important and desirable trait in the pharmaceutical and allied industries; however, a significant difference in phytochemicals has been found within this species (Behura et al., 2002). These factors create a strong need for proactive understanding of the conservation, cultivation, and sustainable usage of this medicinal plant species as well as the production of plants with high and stable contents of expected ingredients.

Improvement of *C. aromatica* is difficult as it does not set seeds. Selection of desirable genotypes with higher yields, curcumin contents, disease and pest resistance, and adaptability can be employed for the development of new cultivars; however, selection methods are limited to mutants arising during vegetative propagation (Sakvi et al., 2001). Thus, there is a need to develop an efficient protocol for the production of true-to-type plantlets with high amounts of expected phytochemicals within a short span of time, and to facilitate germplasm conservation and exchange. An in vitro propagation system provides plants with uniform genetic identity and can ensure clean planting materials (Shatnawi et al., 2011). It provides complementary conservation options for plant species with limited reproductive capacities including a number of rare and endangered species (Cenkci et al., 2007). Reports have been published on in vitro propagation of *Curcuma zedoaria* and *C. longa* using various explants such as rhizome sprout (Loc et al., 2005), bud or shoot-tip (Salvi et al., 2002; Miachir et al., 2004), leaf-base (Salvi et al., 2001), and immature inflorescence (Salvi et al., 2000). However, *C. aromatica* received little attention for in vitro propagation (Nayak, 2000; Miachir et al., 2004; Roy, 2004; Mohanty et al., 2008; Preethi at al., 2010). These reports suggest that micropropagation efficiency of *C. aromatica* requires further improvements. Interestingly, these reports reveal that there is wide variation in growth regulator response within this species. Therefore, a thorough assessment of various growth regulators and other nutrients such as carbon and basal salts is necessary. Since genotypic variation largely affects in vitro propagation, plant materials from wide geographical regions need to be assessed to develop a viable protocol for large-scale commercial micropropagation. In addition, establishment of a highly efficient and reproducible shoot induction system would greatly facilitate *Curcuma* improvement through gene transfer technology. Successful genetic transformation in the close relative *C. longa* has been reported, rarely (Shirgurkar et al., 2006), and it has never been reported in *C. aromatica* until now. This is probably due to lack of efficient regeneration systems. Infecting highly regenerative shoot buds with *Agrobacterium* and the subsequent direct shoot formation would be useful for making transgenic plants. Therefore, this study intends to develop an efficient and repeatable in vitro micropropagation protocol from sprouts of *C. aromatica* that may assist in its conservation and genetic transformation.

Crude extracts of *C. aromatica, C. zedoaria, C. longa*, and *C. malabarica* exhibit antimicrobial activity against gram-positive and gram-negative bacteria and fungi due to the presence of mono-, di-, and sesquiterpenes (Wuthi-udomlert et al., 2000; Wilson et al., 2005; Sharma et al., 2010). In the present study we investigated the antimicrobial activities of some solvent extracts of *C. aromatica* rhizome against some human pathogenic bacteria. Therefore, this study provides a reproducible protocol for high-frequency shoot multiplication of *C. aromatica* using fresh rhizome sprout that could be used for developing uniform clones with high amounts of the expected secondary metabolites.

2. Materials and methods

2.1. Plant materials

Rhizomes of *C. aromatica* collected from the botanic garden of the University of Rajshahi, Bangladesh, were used as the primary source of explants. Sprouts (about 1.0 cm) containing buds covered with several leaf sheaths were excised from the rhizome and used as the explant. Sprouts were washed thoroughly under running tap water followed by immersion in 70% ethanol for 1 min and surface sterilization with 0.1% $HgCl_2$ containing approximately 0.03% (v/v) Tween-20 for 9 min in a laminar airflow cabinet. Then the explants were rinsed 4–5 times with sterile distilled water and placed on different nutrient media.

2.2. Shoot multiplication

The effects of different plant growth regulators (PGRs), carbon sources, basal media, and explant ages on shoot induction of *C. aromatica* were studied under in vitro conditions. To study the effect of PGRs, different kinds

and concentrations of cytokinin benzyl amino purine (BAP) and 6-(α,α-dimethylallylamino)-purine (2iP) were used, either singly or in combination with naphthalene acetic acid (NAA), in full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The media were supplemented with 3% (w/v) sucrose (Table 1). To determine the effect of carbon sources on shoot induction, various saccharides, viz glucose, sucrose, and maltose, were used at different concentrations (2, 3, 4, and 6%; w/v) in the full MS along with 1.0 mg/L BAP. The full- and half-strength MS and modified MS [MS medium containing half of its nitrate (NO_3^-) and ammonium (NH_4^+) salts] were tested to investigate the effect of inorganic nitrogen concentrations on shoot multiplication. The effect of the basal medium was verified by using 3% (w/v) sucrose and 1.0 mg/L BAP. To check the morphogenic potential and efficiency of the protocol for rapid micropropagation, explants were taken from both fresh ex vitro sprouts and in vitro grown plants between 4 and 16 weeks old (secondary explants). The explants were cultured on MS medium supplemented with 3% (w/v) sucrose and 1.0 mg/L BAP for the next 6 weeks.

2.3. Root induction

Microshoots 4–5 cm in length were subcultured on MS medium containing indole-3-butyric acid (IBA) or NAA at 0.1–2.0 mg/L on full- or half-strength MS medium. After adjusting the pH to 5.7, the medium was gelled with 0.8% agar (w/v) (BHD, England) and sterilized by autoclaving at 121 °C for 20 min (1.06 kg/cm^2). Medium was poured into either 25 mm × 150 mm test tubes (12 mL of medium) or 250 mL flasks (50 mL of medium). The plants were subcultured at 4-week intervals from the beginning of the experiment. Cultures were maintained at 25 ± 1 °C under a 16/8-h (light/dark) photoperiod with a light intensity of 45–50 μmol/(m^2 s). The experiment was repeated 3 times for each treatment, which consisted of at least 25 explants.

2.4. Acclimatization and transfer to ex vitro soil

Regenerated plantlets with well-developed root systems were washed carefully in water to remove agar and then transferred to pots containing a mixture of sterile sand, soil, and farmyard manure (1:1:1, v/v/v). Each pot was enclosed in a polyethylene bag after watering and maintained in a growth chamber at 25 ± 1 °C under 16 h of illumination [45 μmol/(m^2 s)] with fluorescent lamps (Alam et al., 2013). Humidity was reduced gradually by creating a hole (0.5 cm) in the bag and increasing its size every 3–5 days over a period of 2–3 weeks. The surviving plantlets were transferred to large pots for further development.

2.5. Preparation of plant extract for antimicrobial activities assay

Dried and powdered rhizome of field-grown plants was soaked separately in petroleum ether and chloroform (1:10, w/v) for 4–5 days at room temperature with occasional shaking. After filtration of the extracted solutions (by Whatman No. 1 filter paper), they were concentrated to dryness in a rotary evaporator at 37 °C. For aqueous extraction, fresh rhizome was ground to paste using a mortar and pestle and soaked in sterile distilled water (1:10, w/v) in a conical flask, maintained for 24 h at gentle agitation, and then filtered off using sterile filter paper. This extract was subjected to a water bath for evaporation to obtain the crude extract. Collected solid residues (crude extract) were stored at 4 °C and were freshly dissolved in the respective solvents prior to screening for antimicrobial activity.

2.6. Tested bacterial species

Two gram-positive bacteria, *Bacillus subtilis* (NCIM 2063) and *Staphylococcus aureus* (NCIM 2127), and 3 gram-negative bacteria including *Pseudomonas aeruginosa* (NCIM 5029), *Shigella sonnei*, and *Shigella dysenteriae* (clinical isolates) were used to evaluate antimicrobial activities. The bacteria were obtained from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The bacteria were cultured on a nutrient agar medium at 37 °C and maintained by subculturing periodically on an agar slant at 4 °C.

2.7. Evaluation of antimicrobial activity

Antibacterial activity was tested by disk-diffusion method (NCCLS, 1998). Extracts were prepared by reconstituting the extracts in the respective solvents (petroleum ether, chloroform, or sterile distilled water). Then, 100 μL of the 24-h-old broth culture from each tested bacterium (McFarland standard 0.5) was spread on a nutrient agar plate. Autoclave sterilized filter paper disks (6 mm diameter) were soaked with 50 μL of dissolved chloroform or aqueous extract (3.0 mg/mL) or 50 μL of petroleum ether extract (1.0 mg/mL) and allowed to dry for a few minutes and then placed on top of the seeded plate. Standard antibiotic disks (Hi-Media, Mumbai, India) of tetracycline and gentamycin (10 μg) served as the positive controls. The assay plates were incubated at 37 °C for 24 h. The diameter of the zones of inhibition around each of the disks (disk included) was considered a measure of the antimicrobial activity. Each experiment was carried out in triplicate, and the mean diameter of the inhibition zone was recorded. The extracts, which showed antimicrobial activity in the disk, were subjected to MIC assay (Wilson et al., 2005). Serial 2-fold dilutions of the extracts ranging from 0.004 to 4 mg/mL were prepared in 2 mL of nutrient broth. Then 40 μL of the test organism (0.5 McFarland turbidity standards) was added and incubated for 24 h. The MIC values were interpreted as the lowest concentrations of the extracts that showed clear fluid with no development of turbidity. Blanks and positive controls were also included.

2.8. Statistical analysis

Statistical analysis of the data was performed using SPSS software (version 17.0). One-way analysis of variance (ANOVA) was used to evaluate the effect of growth regulators on shoot formation and root induction. Within the treatment groups, data were analyzed by one-way ANOVA, followed by Tukey's honestly significant difference (HSD) test as a post hoc comparison. Results were expressed as mean ± SE, and differences in mean values are considered to be statistically significant at $P < 0.05$. The number of shoots and roots in response to growth regulators was subjected to polynomial fitting using Origin (v. 8.5).

3. Results and discussion

3.1. Surface sterilization

Successful surface sterilization is one of the most critical steps of in vitro culture, especially when rhizomes are used. Freshly sprouted buds were used as the explant for initiation of in vitro culture. By using a 0.1% mercuric chloride solution, more than 70% of explants were free of contamination after 4 weeks of culture (data not shown). Our results are similar to those reported by Loc et al. (2005) in *Curcuma zedoaria* and by Shatnawi et al. (2011) in *Vitis vinifera*.

3.2. Effect of plant growth regulators on shoot proliferation

At the beginning, the effects of different cytokinins and their levels and cytokinin in combination with auxins were tested for induction of new shoots from the bud explants. The overall dose response pattern of BA and 2iP is given in Figure 1. It was observed that within 4–10 days of inoculation (Figure 2A) explants showed swelling with rupturing of the scale leaves and directly produced single or multiple shoots according to the plant growth regulator used (Figure 2B). BAP was the most effective for high frequency of multiple shoot induction (Table 1). The highest numbers of shoots (13.20 shoots per explant) were obtained on MS medium supplemented with 1.0 mg/L BAP (Table 1), while the maximum shoot length (4.95 mm) was obtained in medium supplemented with 2.0 mg/L 2iP. The maximum proliferation rate (95.3%) was obtained on medium supplemented with 1.0 mg/L BAP concentration, which is higher than the control (Figure 2C).

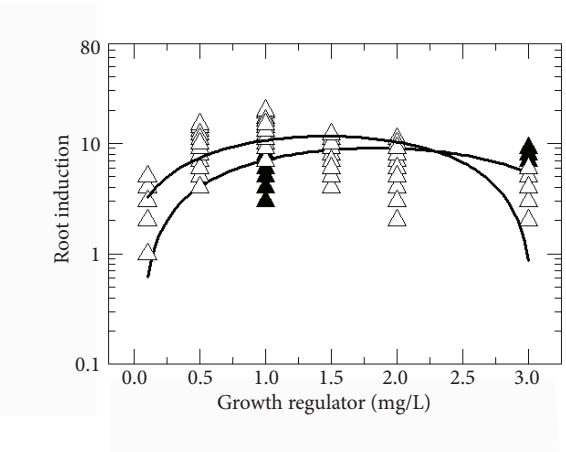

Figure 1. Dose response pattern of BA (Δ) and 2iP (▲) on in vitro shoot induction (shoot number) from the rhizome sprout explant in *C. aromatica*. Data were recorded after 6 weeks of culture on MS medium using 3% w/v sucrose.

When used alone, 2iP also induced multiple buds initially; however, their growth was not vigorous and produced unhealthy and stunted shoots. The addition of NAA did not improve the growth and development of shoots (Table 1). The maximum percentage of shoot initiation suggests that shoot induction from sprouts of *C. aromatica* requires a moderate concentration of cytokinin only. In the current study the maximum number of microshoots (an average of 13 shoots per explant) was obtained in MS medium supplemented with 1.0 mg/L BAP. BAP induced more shoots than 2iP or other auxin-cytokinin combinations, as is evident from ANOVA and multiple comparison tests. These data also showed that the dose response within each growth regulator is also distinctly different. Similar responses have been described in other *Curcuma* genera and other medicinal plants (Nayak, 2000; Miachir et al., 2004; Loc et al., 2005; Thomas and Shankar, 2009). In contrast, Roy and Raychaudhurp obtained the highest number of shoots (~4 shoots) with a kinetin (5 mg/L) and NAA (4 mg/L) combination in *C. aromatica* using nodal explants (Roy, 2004), while a combination of BA (2.22 μM) and NAA (2.69 μM) is recommended by Preethi et al. (2010) for this species. The differential response of *C. aromatica* in PGR-response and shoot induction efficiency is probably due to genotype, as the plant material was obtained from diverse geographical locations. It is possible that specific genetic combinations in some genotypes are more responsive to a particular type of morphogenesis than others. Our results demonstrate that micropropagation protocols of *C. aromatica* need to be refined to better understand the role of the genotype for commercialization. In long-term breeding programs, genotypes amenable to in vitro cloning need to be selected over the less responsive genotypes.

The PGR-free MS medium led the subcultured shoots to elongate with only some weak root development (Figure 2D), whereas new shoot induction rarely occurred. Thus, an additional culture in rooting medium was required (Figure 2E) for successful establishment in soil conditions (Figure 2F). A comparative analysis of micropropagation potential between the in vitro grown (16-week-old) plantlets and freshly developed ex vitro sprouts showed a similar shoot induction rate (data not shown). This

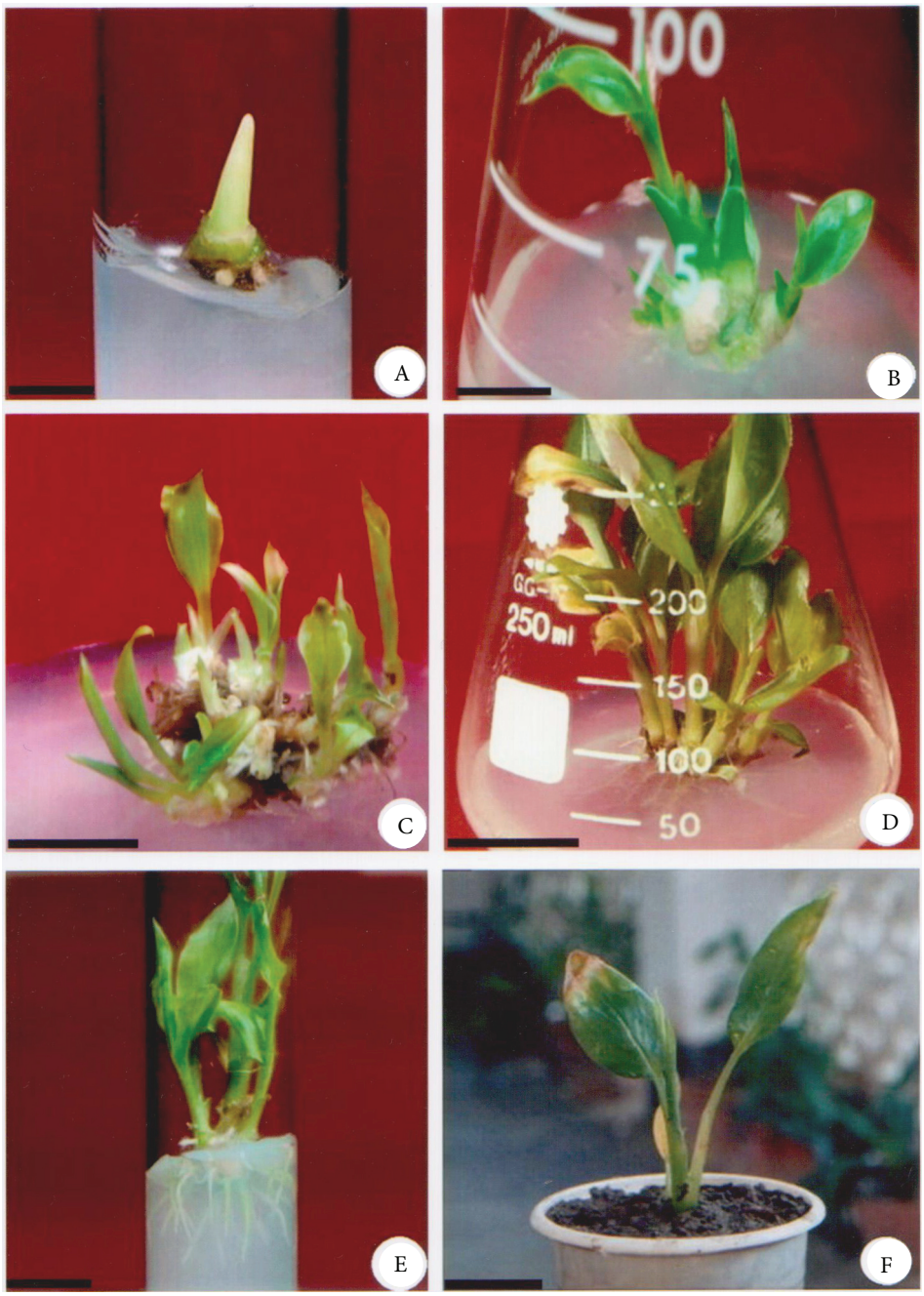

Figure 2. In vitro propagation via shoot morphogenesis in *C. aromatica* (A–F). A: rhizome sprout after 7 days of inoculation. B and C: multiple shoots induced from the rhizome sprout explants after 3 and 6 weeks, respectively. D: a cluster of shoots after transfer to PGR-free medium. E: root development of in vitro grown plantlets. F: an acclimatized plant in soil. Bars represent 1 cm (A, E) or 2 cm (B, C, D, F).

suggests that the age of in vitro explants does not influence shoot induction much. In support of our investigation, continuous shoot production from in vitro established plants has also been observed in other plants, including *C. longa* (Sharma and Singh, 1997; Prathanaturarug et al., 2003).

3.3. Effect of different carbon sources on microshoot development

A comparative study of plantlets grown on sucrose, glucose, and maltose, which were supplemented with the same growth regulators, indicated that 3% (w/v) sucrose gave a higher shoot number and length (Figure 3). Carbon

Table 1. Effects of different concentrations and combinations of BAP, 2iP, and NAA on multiple shoot induction from the rhizome bud explant in *C. aromatica* (data were recorded after 6 weeks of culture on MS medium using 3% w/v sucrose). Values are the means ± standard error. Different superscripts in the same column indicate significant differences within treatments (Tukey's HSD test, $P < 0.005$).

Plant growth regulator (mg/L)			Shoot induction rate (%)	Mean number of shoots per culture ($\bar{X}$± SE)	Mean length (cm)
BA	2iP	NAA			
0.1			40.08	2.86 ± 0.54^{ab}	3.42 ± 0.71^{ab}
0.5			78.92	9.85 ± 0.36^{h}	3.60 ± 0.89^{ab}
1.0			95.33	13.20 ± 0.69^{i}	3.90 ± 0.32^{ab}
1.5			70.50	8.15 ± 0.48^{gh}	4.10 ± 0.16^{ab}
2.0			58.36	6.25 ± 1.10^{defg}	4.33 ± 0.27^{ab}
3.0			32.45	3.20 ± 0.45^{abc}	3.65 ± 0.51^{ab}
	0.1		45.36	1.30 ± 0.87^{a}	2.60 ± 0.87^{a}
	1.0		63.40	5.85 ± 0.41^{def}	3.50 ± 0.46^{ab}
	2.0		70.85	7.10 ± 0.97^{fg}	4.95 ± 0.51^{b}
	3.0		58.91	4.92 ± 0.71^{cd}	4.23 ± 0.72^{ab}
0.5		0.1	53.50	3.80 ± 0.39^{bc}	3.88 ± 0.49^{ab}
1.0		0.1	75.75	6.90 ± 0.36^{efg}	4.10 ± 0.76^{ab}
1.0		0.5	60.33	5.10 ± 0.78^{cde}	4.95 ± 0.82^{b}
2.0		0.5	42.93	2.33 ± 0.61^{ab}	3.15 ± 0.79^{a}

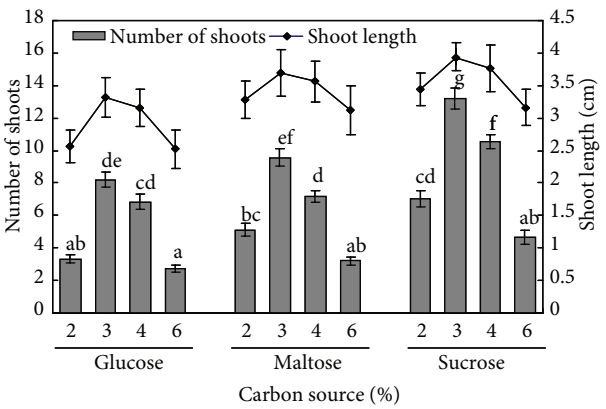

Figure 3. Effect of different carbon sources and concentrations on in vitro shoot induction of *C. aromatica*. Experiment was conducted using MS basal salt with 1.0 mg/L BAP. Data were recorded after 6 weeks of culture. Different letters on the bars indicate significant differences within treatments (Tukey's HSD test, $P < 0.005$).

sources and their concentrations distinctly influenced shoot multiplication, and 3% (v/v) sucrose was found to be the optimum with respect to shoot number. Shoot number gradually decreased in response to both lower and higher levels of sucrose. The majority of in vitro studies have concluded that sucrose supports near optimal rates of growth, and it is relatively inexpensive (Swedlund and Locy, 1993; Shatnawi et al., 2007; Shatnawi et al., 2011). Mello et al. (2001) observed a typical growth curve based on dry matter accumulation and recommended sucrose as the best carbon source. The preference for sucrose for in vitro cultures of most plant species, including *Curcuma*, can be attributed to its properties as a nonreducing sugar; it is inert with no further enzymatic conversions, even in long-distance transport (Kato et al., 2007). On the other hand, a high level of sucrose reduced shoot growth. This might be due to the decreased water potential of the medium (Adelberg, 2010). In addition, increased sucrose hydrolysis is involved in the enzymatic process of sucrose absorption and/or uptake and the subsequent release of glucose and fructose to the cytosol. This cycle reduced dry matter accumulation in in vitro plants (Riek et al., 1997). Salvi et al. (2001) found that glucose is the most efficient carbon source for shoot multiplication in *C. longa*.

3.4. Effect of type of media on microshoot development

Considering the strength of the MS salt, full-strength MS medium was better for shoot multiplication than half MS or modified MS (Figure 4), which indicates that the full ionic concentration is useful for optimum shoot induction in *C. aromatica*. Similar results have been reported in *Kaempferia galanga* (Shirin et al., 2000) and *Aframomum corrorima* (Tefera and Wannakrairoj, 2004). MS medium contains a higher amount of nitrogen than the other

media, in 2 forms: NO_3^- and NH_4^+. Nitrogen is involved in the synthesis of several principal components of plant cells, including amino acids, proteins, nucleic acids, and chlorophyll, that are indispensable to plant growth and development; thus, its deficiency may result in reduced growth in some plants (Tefera and Wannakrairoj, 2004).

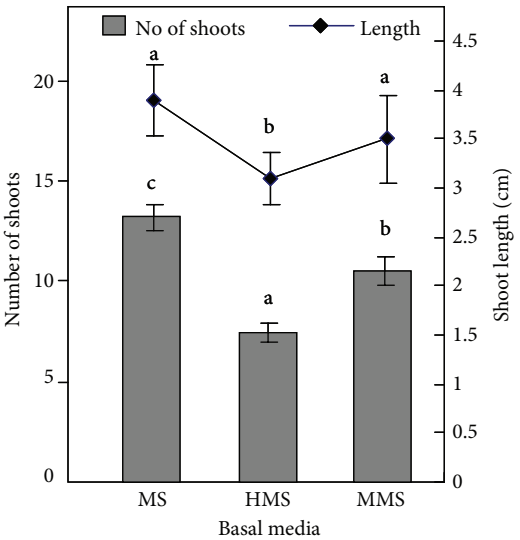

Figure 4. Effect of basal medium on in vitro shoot induction of *C. aromatica*. Experiment was conducted using 1.0 mg/L BAP and 3% (w/v) sucrose. Data were recorded after 6 weeks of culture. MS: full MS, HMS: half MS, and MMS: modified MS, as described in the materials and methods section. Different letters on the bars indicate significant differences within treatments (Tukey's HSD test, $P < 0.005$).

3.5. In vitro root formation and acclimatization

With an optimum BAP concentration, root formation was negligible. Nadgauda et al. (1978) reported that root initiation and development declined with increasing levels of BAP in a turmeric tissue culture. Effective root proliferation was not observed in the shoot multiplication medium; therefore, developed microshoots were cultured separately for root induction. This result is similar to the previous finding by Shatnawi et al. (2011). Root initiation was achieved from the bases of shoots after incubation on media containing NAA or IBA in various concentrations. The dose response pattern is shown in Figure 5. Maximum root number was obtained with the addition of NAA at 0.5 mg/L with an average 3.5 cm root length, compared with 0.1 mg/L NAA (2.23 cm) (Table 2). Proliferation rate increased up to 100% with 0.5 mg/L NAA. Shoot length was affected by the use of NAA or IBA compared with low concentrations. From the results (Table 2), it can be inferred that 0.5 mg/L NAA produced optimum root induction and development. Our result is similar to previous reports on various members of Zingiberaceae. Taken together, these results suggest that low concentrations of NAA and IBA were effective in inducing rooting in *C. aromatica,* and the development of roots was quite similar at the different auxin concentrations. This has also been found in previous studies, in which in vitro shoots of *C. zedoaria* and grapevine were reported to root with a supplement of NAA or IBA (Loc et al., 2005; Shatnawi et al., 2011). On the other hand, simultaneous root formation in shoot formation medium has been reported in *C. aromatica* (Nayak, 2000) and several other Zingiberaceae plants including *C. longa* (Sharma and Singh, 1997; Salvi et al.,

Table 2. Effects of different concentrations of IBA and NAA on root development of in vitro grown micro-shoots of *C. aromatica*. Data were recorded after 4 weeks of culture on MS medium using 3% (w/v) sucrose. Values are the means ± standard error. Different superscripts in the same column indicate significant differences within treatments (Tukey's HSD test, $P < 0.005$).

Plant growth regulator (mg/L)		Root induction rate (%)	Mean number of roots per culture ($\bar{X}$± SE)	Mean length (cm)
NAA	IBA			
0.1		65.66	4.42 ± 0.23^{b}	2.23 ± 0.29^{a}
0.5		100.0	9.40 ± 0.98^{e}	3.52 ± 0.52^{bc}
1.0		92.08	7.33 ± 0.81^{d}	3.10 ± 0.52^{abc}
2.0		74.33	5.20 ± 0.85^{bc}	2.36 ± 0.34^{ab}
	0.1	50.60	2.36 ± 0.29^{a}	2.05 ± 0.69^{a}
	0.5	89.60	6.80 ± 0.62^{cd}	3.46 ± 0.63^{c}
	1.0	75.95	4.33 ± 0.32^{b}	3.18 ± 0.32^{abc}
	2.0	82.33	5.72 ± 0.26^{bcd}	2.75 ± 0.52^{abc}

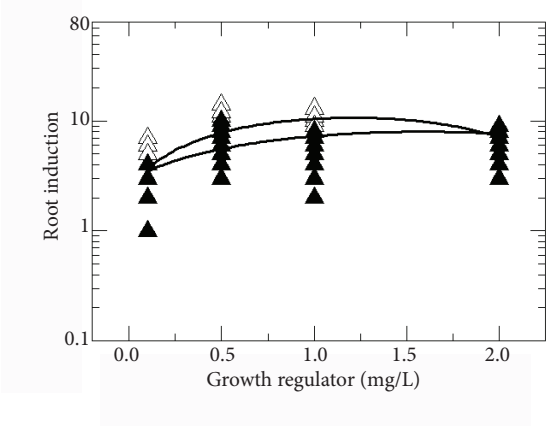

Figure 5. Dose response pattern of NAA (Δ) and IBA (▲) on in vitro root induction (root number) of in vitro grown *C. aromatica* micro-shoots. Data were recorded after 4 weeks of culture on MS medium using 3% (w/v) sucrose.

2002) and *Kaempferia galanga* (Shirin et al., 2000). Such a difference in root induction response can be explained by the complex interactions between the endogenous and exogenous levels of hormones during in vitro culture. Molecular studies showed that auxin and its polar transport are the central regulators of lateral root formation. It has been reported that auxin-dependent nuclear signaling is necessary for lateral root formation (Ditengou et al., 2008). Optimum rooting response using IBA and NAA has been reported for several other medicinal plants including *Syzygium francissi* (Shatnawi et al., 2004), pear (Shatnawi et al., 2007), *Achillea millefolium* (Shatnawi, 2013), and *Stevia rebaudiana* (Zayova et al., 2013). In the current study a protocol to obtain roots from in vitro microshoot culture of *C. aromatica* was developed.

3.6. Antibacterial activity and minimum inhibitory concentrations of rhizome extracts

The quest for antimicrobial chemicals in plants has received increasing attention (Arslan et al., 2012; Erel et al., 2012; Karakaş et al., 2012; Orhan et al., 2012). The antimicrobial activities of 3 solvent extracts of *C. aromatica* rhizome, compared with those of gentamycin and tetracycline, are summarized in Table 3. An assay of the extracts revealed that petroleum ether and chloroform extracts showed varying degrees of inhibition against pathogenic bacteria, while an aqueous extract did not show marked zones of inhibition. In general, petroleum ether showed higher activity than chloroform extracts and produced inhibition zones ranging from 8 to 16 mm in diameter at a concentration of 1.0 mg/mL, whereas chloroform extracts were effective at a higher concentration (3.0 mg/mL) (Figure 6).

The minimum inhibitory concentration (MIC) values of active extracts were determined by the broth dilution method and are presented in Table 4. The MIC value of

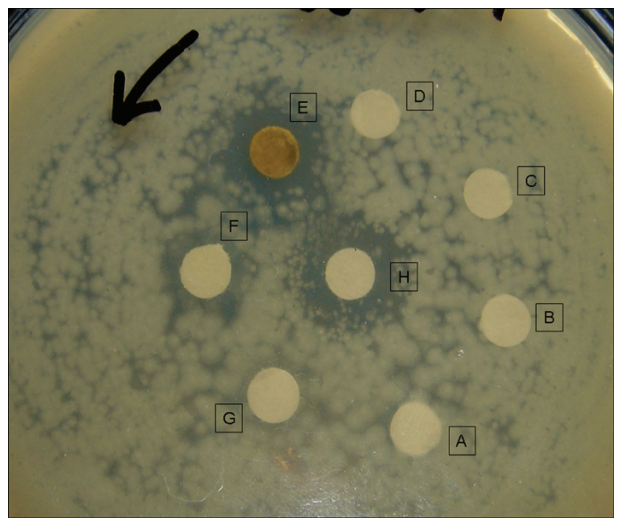

Figure 6. A representative plate of the disk diffusion assay for *Staphylococcus aureus*. A: chloroform solvent blank, B: aqueous extract, C: petroleum ether solvent blank, D: petroleum ether extract (0.004 mg/mL), E: petroleum ether solvent (1 mg/mL), F: chloroform extract (3 mg/mL), G: chloroform extract (0.004 mg/mL), and H: antibiotic gentamycin (10 µg/disk).

Table 3. Antibacterial activity of *C. aromatica* rhizome extracts against 5 pathogenic bacteria species. Values are the means ± standard error. Different superscripts in the same row indicate significant differences within treatments (Tukey's HSD test, $P < 0.005$).

Extract / Bacteria	Zone of inhibition (mm)			
	Petroleum ether	Chloroform	Tetracycline	Gentamycin
Staphylococcus aureus	15.3 ± 0.88^{ab}	11.7 ± 0.66^{a}	23.0 ± 1.73^{c}	18.0 ± 0.57^{b}
Bacillus subtilis	16.3 ± 0.88^{b}	10.0 ± 1.52^{a}	22.0 ± 1.00^{bc}	24.0 ± 1.52^{c}
Pseudomonas aeruginosa	11.7 ± 0.66^{a}	8.3 ± 0.90^{a}	18.3 ± 1.20^{b}	19.0 ± 1.15^{b}
Shigella sonnei	9.7 ± 0.88^{a}	8.7 ± 0.70^{a}	16.0 ± 1.52^{b}	18.3 ± 1.20^{b}
Shigella dysenteriae	10.0 ± 0.57^{a}	8.0 ± 0.60^{a}	14.3 ± 0.66^{b}	21.6 ± 1.20^{c}

Table 4. Minimum inhibitory concentration (MIC) of petroleum ether and chloroform solvent extracts against 5 pathogenic bacteria.

Bacteria \ Extract	MIC (mg/mL)	
	Petroleum ether	Chloroform
Staphylococcus aureus	0.06	0.13
Bacillus subtilis	0.03	0.06
Pseudomonas aeruginosa	0.25	0.50
Shigella sonnei	0.25	0.25
Shigella dysenteriae	0.13	0.25

extracts ranged from 0.03 to 0.5 mg/mL. Petroleum ether extracts had lower MIC values compared with chloroform extract against the tested strains. Higher degrees of inhibition were observed against gram-negative bacteria, especially *Bacillus subtilis* and *Staphylococcus aureus.* Antimicrobial activities of extracts of several zingibers, such as *C. zedoaria, C. longa, C. malabarica,* and *C. amada*, against gram-positive and -negative bacteria and fungi have been reported in many studies (Yoshioka et al., 1998; Negi et al., 1999; Mujumdar et al., 2000; Wuthi-udomlert et al., 2000; Wilson et al., 2005).

In conclusion, *C. aromatica* is becoming endangered at an alarming rate in the wild because of ruinous over-harvesting for pharmaceuticals with little or no regard for the needs of the future. Considering the potential of the plant, the culture system described here is efficient and has the potential for application to large-scale multiplication since multiple shoots were directly generated without the callusing passage, and somaclonal variations could be minimal. In addition, the current micropropagation system yielded a higher number of plantlets than earlier studies. Along with micropropagation, the present study revealed that the field grown rhizome extracts of *C. aromatica* successfully inhibit the growth of several gram-positive and -negative bacteria. This study finds good evidence for promising antimicrobial components in the *C. aromatica* rhizome that could be useful in developing antimicrobial substances in the medicinal industries.

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