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Efficient Procedure for *In vitro* Microrhizome Induction in *Curcuma longa* L. (Zingiberaceae) – A Medicinal Plant of Tropical Asia

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Abstract

An improved *in vitro* microrhizome induction system in *Curcuma longa* L. has been developed. Freshly sprouted axillary buds from underground rhizomes were used as initial explants and multiplied through established *in vitro* systems. Multiplied shoots were excised and subcultured on hormone free medium for four weeks to induce microrhizome. Effects of light, sucrose and growth regulators on *in vitro* microrhizome production have been studied. Nine per cent sucrose was found to be the most suitable for microrhizome production, when incubated in the dark. Various concentrations of BA and Kn, and NAA were tested. BA (12.0 µM) and NAA (0.3 µM) were found suitable for the induction of microrhizomes. Larger microrhizomes performed better under *in vivo* conditions and developed shoots readily, when they were transferred directly from *in vitro* to soil without acclimatization. Microrhizomes produced under *in vitro* conditions can be stored and transported easily which are advantageous for *in vitro* shoot multiplication.

Introduction

Curcuma longa L. (turmeric) is a herbaceous plant of the Zingiberaceae (Purseglove 1972). The plant is an economically important cultivated species in South and South-East Asia that has been used since the Vedic age as a spice, herbal medicine, dyeing agent and as a cosmetic (Salvi et al. 2000, Shirgurkar et al. 2001). The chemical composition of *C. longa* has been thoroughly investigated and a number of different active substances that exhibit germicidal, aromatic, carminative, antihelmentic, antioxidant, anti-tumour,

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cholesterol lowering and neuroprotective activities have been found (Cao et al. 2001, Jitoe et al. 1992, Kikuzaki and Nakatani 1993, Masuda et al. 1993, Sasaki et al. 2002). Turmeric is a sterile triploid plant that is vegetatively propa-gated by means of underground rhizomes. The rate of rhizome multiplication in this plant is very low (only six - ten times) with yield ranging from 15 to 25 tons/hectare (Balachandran et al. 1990). A considerable amount (10 - 20%) of rhizomes of the total yield is required for the next cropping year (Shirgurkar et al. 2001). Maintenance of such a huge amount of seed-rhizomes for annual planting is expensive and labour intensive. Moreover, many diseases and pests, particularly soft rot of turmeric caused by *Pythium* spp. and bacterial wilt are consistently threatening the germplasm of this important medicinal plant (Balachandran et al. 1990, Nayak 2000, Salvi et al. 2001 and 2002, Shirgurkar et al. 2001).

Recently *in vitro* formation of storage organs such as bulbs, corms, tubers and rhizomes came into focus because these kinds of propagules can be directly transferred to the field without any acclimatization or hardening. In addition, these organs can be easily transported internationally as they do not require any culture medium or any other special measures to prevent contamination. Although a number of reports are available for different plants such as *Solanum tuberosum* L. (Abbott and Belcher 1986, Gopal et al. 1998, Garner and Blake 1989, Hoque et al. 1996, Vreugdenhil et al. 1998), *Dioscoria composita* Hemsl. (Alizadeh et al. 1998), *Bunium persicum* (Grewal 1996), *Trillium erectum* L. and *T. grandiflorum* (Michx.) Salisb. (Pence and Soukup 1993) and *Lachenalia* (Slabbert and Niederwieser 1999), only a few reports on turmeric (Nayak 2000, Shirgurkar et al. 2001, Sunitibala et al. 2001) and ginger (Sharma and Singh 1995, Nirmalbabu et al. 1994) are available. However, further improvement of the protocol is essential to obtain larger and more vigorous microrhizomes since the survival of small microrhizomes is very low and small rhizomes normally produce unhealthy, stunted plants (Shirgurkar 2001). There is also a disagreement between the reports of Nayak (2000) and Shirgurkar (2001). While the former described BA as a growth promoter, the latter mentioned it as a growth inhibitor.

The present investigation was carried out to see the effects of *in vitro* culture conditions such as sucrose concentration, strength of MS medium and light illumination on large scale microrhizome induction in *C. longa* L. The effect of BA and Kn as well as NAA were also studied since they are described as effective growth regulators for the genus *Curcuma* (Balachandran et al. 1990, Dekkers 1991, Nadgauda et al. 1978, Salvi et al. 2002, Shirgurkar et al. 2001, Sunitibala et al. 2001, Yasuda et al. 1988).

Materials and Methods

Establishment of contamination free cultures: Underground rhizomes of *C. longa* L. collected from Bangladesh National Herbarium (DACB), Mirpur, were cultivated in the glasshouse of the Institute of Botany, University of Hannover, Germany to allow sprouting of buds. Sprouted immature shoots (ca. 1 cm long) were collected and used as the source of explants. Immature buds were cleaned with running tap water and then washed with detergent (Tween-20) for 5 min, subsequently rinsed thoroughly under running tap water for 5 min. Explants were then immersed in 70% ethanol for 30 - 40 sec before incubation in disinfectant (0.1% $HgCl_2$ to which two - three drops of Tween-20/100 ml were added). Under sterile conditions, $HgCl_2$ solution was decanted and the explants were rinsed five - six times with sterile distilled water. Sterilized sprouts were then dissected to remove the outer few layers of leaf sheaths under aseptic conditions. Excised buds were initially cultured on MS basal medium supplemented with 6 µM BA and 0.3 µM NAA, and 3% sucrose. The medium was solidified with 0.8% agar (Duchefa, NL) after adjusting the pH to 5.8 and sterilization by autoclaving at 121_C (1.06 Kg^{-1} m^{-2}) for 20 min. About 25 ml of the medium (M1) were dispensed into the sterile plastic, `De Wit' culture tubes (Duchefa, NL). Initial explants were cultured on this shoot induction medium in 16 h light (white fluorescent light with 50 µM m^{-2} s^{-1}) and 8 h darkness in order to obtain contamination free cultures since contamination of the explants taken from underground rhizomes of *C. longa* had been a major problem (Balachandran et al. 1990, Salvi et al. 2002).

After six weeks, contamination-free explants were transferred to large vials containing the same medium for another six weeks for shoot multiplication. Vigorous multiple shoot regeneration was observed surrounding the initial cultures. These shoots were excised and transferred to M2 medium (M1 medium devoid of growth hormones) and cultured for another four weeks to avoid carry-over effects of growth regulators, and used in all subsequent experiments using liquid medium in a 100-ml flask.

Effects of sucrose: BA, Kn, NAA and MS salts - An experiment was carried out to determine the effect of the concentration of sucrose using M3 medium (M1 medium minus of agar and 0.75∇ MS salts) with 1, 3, 5, 7, 9 and 11% sucrose treatments. All treatments were investigated under both complete darkness and 16 h photoperiod (50 µM m^{-2} s^{-1}). Sixteen combinations (Table 1) with different concentrations of BA and Kn alone or in combination with 0.3 µM NAA were investigated under complete darkness using M4 medium (M3 medium modified with 9% sucrose). Effects of different concentrations of NAA (0.3, 0.6, 0.9 and 1.2

µM) were also studied using M5 medium (M4 medium modified with 12 µM BA). In order to optimize the strength of MS basal medium, explants were cultured on M6 medium (M5 medium modified with 0.3 µM NAA) with different strengths of MS salts: 1.00∇, 0.75∇, 0.50∇ and 0.25∇.

Development of plantlets and glasshouse evaluation: In vitro produced microrhizomes were isolated after 60 days. Harvested microrhizomes were directly transferred to the soil for plantlet development. These potted rhizomes were maintained in the glasshouse at ambient temperature with 70 - 80% relative humidity and about 16 h photoperiod. After four months of culture, plants developed from three different sizes of microrhizomes [0.5 – 1.0 cm (small), 1.1 – 2.0 cm (medium) and > 2.0 cm (large)] were evaluated using various morphological characters.

Data analysis: Results presented in the tables and in the figures are the pooled means ± standard errors (SE) of two repeated experiments each with 15 replications with an exception in which ten replicates were used without any repetition (Table 3). The test of statistical significance was done by applying Tukey's test at 5% level using SAS statistical software, Release 8 (SAS Institute Inc., Cary, NC).

Results and Discussion

Establishment of initial culture and multiplication: Establishment of conta-mination-free *in vitro* cultures of *C. longa* was already described by other groups (Balachandran et al. 1990, Dekkers 1991, Nadgauda et al. 1978, Salvi et al. 2002, Shirgurkar et al. 2001, Sunitibala et al. 2001, Yasuda et al. 1988). Many authors argued that the contamination of underground rhizomes was very high and that the establishment of contamination free cultures was difficult. Therefore, in this study, the established method boosted contamination-free cultures and multiplication. We had better results by incorporating Tween-20 with 0.1% $HgCl_2$ as a wetting agent to reduce surface tension and also to allow better surface contact. Under these conditions more than 70% explants remained contamination-free until the next subculture. It seems that use of Tween-20 helped get a higher percentage of contamination-free explants since it allowed better surface sterilization. After four weeks, contamination free explants were multiplied using our established protocol containing MS basal medium (pH 5.8), 3% sucrose, 0.8% agar and a low concentration of BA (6 µM) and NAA (0.3 µM). Multiplied shoots were then cultured on plant growth regulator (PGR) free medium to avoid carry-over effects. Fig. 1 (a - e) represents the different developmental stages of *in vitro* microrhizome induction in *C. longa* and the field level adaptation.

Effects of sucrose: In order to develop an optimised standard protocol, effects of different concentrations of sucrose were investigated under fully dark and 16 h photoperiod (white fluorescent light with 50 µM m^{-2} s^{-1} light intensity). It was observed that sucrose plays a significant role in the size and

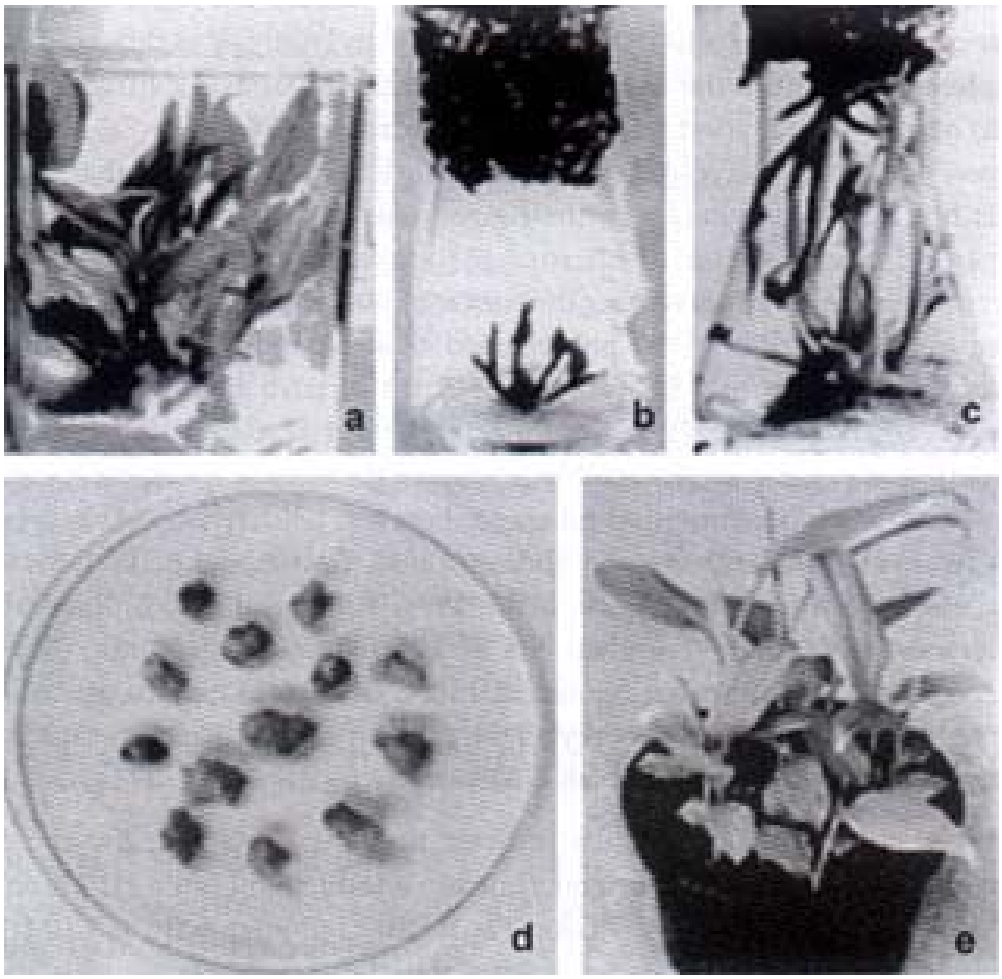

Fig. 1a. Multiple shoots obtained from shoot multiplication medium after four - six weeks, b. Explants cultured on liquid medium for microrhizome induction, c. After 60 days microrhizome is developed along with a clump of roots, d. Collected microrhizomes, e. Shoot development and establishment in the soil.

number of microrhizomes in *C. longa*. In nine per cent sucrose, the production was highest (8.3 ± 0.32). The largest (0.88 g ± 0.03) healthy microrhizomes were obtained in the dark, while incubation under the light reduced the number (8.3 ± 0.35) and size (0.82 ± 0.03 g) slightly, but not significantly. Seven per cent

sucrose reduced both number and size but it was not statistically significant. However, both lower (0 - 5%) and higher (11%) concentrations of sucrose had significant inhibitory effects on microrhizome production. The results of the present investigation supports the reports of Shirgurkar et al. (2001), Sunitibala et al. (2001) and Nayak (2000) who obtained optimum micro-rhizome induction in *Curcuma* using 6 - 9% sucrose. They also stated that a lower concentration of sucrose decreased the size and number of rhizomes or even completely prevented the induction of any microrhizomes. Shirgurkar et al. (2001) obtained the highest number of microrhizomes (5.6 ± 0.8 - 7.0 ± 1.1) at 6% sucrose, while at 8% sucrose they found a reduction in the number (5.6 ± 0.5 – 5.8 ± 1.1) but a slightly increase in their size. Nayak (2000) observed that at an obligatory concentration of sucrose (3%), plants could not develop any microrhizomes even by increasing the concentration of BA from 1 to 7 mg/l or by increasing the duration of the photoperiod. Sharma and Singh (1995) reported that a concentration of 7.5% was required for *in vitro* rhizome induction in *Zingiber officinale* Rosc. In many other reports it is also stated that a high concentration of sucrose remarkably promotes the *in vitro* formation of storage organs such as bulbs, corms and tubers (Abbott and Belcher 1986, Alizadeh et al. 1998, Arora et al. 1996, Dantu and Bhojwani 1987, Garner and Blake 1989, Gopal et al. 1998, Grewal 1996, Hoque et al. 1996, Kim et al. 2003, Slabbert and Niederwieser 1999 and Vreugdenhil et al. 1998). The enhanced rate of *in vitro* organ formation with increasing concentration of sucrose may be attributed to the presence of high carbon energy in the form of sucrose since storage organs mostly store carbohydrates (Nayak 2000).

Effects of BA and Kn: Results indicate that the PGRs BA, Kn and NAA had significant effects on *in vitro* microrhizome induction (Table 1). Among the 16 different combinations of PGRs, 12 µM BA together with 0.3 µM NAA exhibited a better response than any other treatments in terms of mean number (8.1 ± 0.36) and the weight (0.67 ± 0.03 g) of microrhizomes. Nayak (2000) and Sharma and Singh (1995) also reported that 5 mg/l BA in *C. aromatica* Salisb. and 8 mg/l BA in ginger, respectively enhanced microrhizome production. However, Shirgurkar et al. (2001) stated that BA had an inhibitory effect on *in vitro* microrhizome production in turmeric, though at the lowest level of 4.4 µM, it did not have much adverse effects on the average number, weight and yield of microrhizomes, but at the maximum concentration of 35.2 µM BA microrhizome production was completely inhibited. In the present study, Kn alone or in presence of NAA did not show any promising result, producing only up to 4.5 ± 0.41 microrhizomes and weighting 0.39 ± 0.02 g, when 12.0 µM BA and 0.3 µM NAA were added to the basal medium. However, Sunitibala et al.

(2001) reported that Kn (1 mg/l) is suitable for *in vitro* rhizome induction in *C. longa* L. Grewal (1996) also stated that Kn is an efficient cytokinin for enhancing microtuber induction of somatic embryos of *Bunium persicum*.

Table 1. Effects of BA, Kn alone or in presence of 0.3 µM NAA on *in vitro* microrhizome formation in *Curcuma longa* L.

BA/Kn	NAA (µM)	Number (SE)	Weight (g) (SE)
Control	-	2.7 (0.21)de	0.31 (0.02)fg
3 µM BA	-	3.9 (0.32)bcde	0.32 (0.02)efg
6 µM BA	-	4.0 (0.29)bcde	0.36 (0.01)cdefg
12 µM BA	-	5.6 (0.35)b	0.48 (0.03)b
18 µM BA	-	5.0 (0.45)bc	0.45 (0.03)bcd
3 µM BA	0.3	5.0 (0.34)bc	0.45 (0.02)bcd
6 µM BA	0.3	5.3 (0.45)b	0.47(0.02)bc
12 µM BA	0.3	8.1 (0.36)a	0.67 (0.03)a
18 µM BA	0.3	4.0 (0.41) bcde	0.44 (0.03)bcde
3 µM Kn	-	3.1 (0.32)de	0.32 (0.02)efg
6 µM Kn	-	3.5 (0.31)cde	0.38 (0.02)bcdefg
12 µM Kn	-	3.9 (0.33)bcde	0.39 (0.02)bcdefg
18 µM Kn	-	2.9 (0.24)de	0.34 (0.02)defg
3 µM Kn	0.3	4.4 (0.34)bcd	0.42 (0.02)bcdef
6 µM Kn	0.3	4.1(0.48)bcde	0.35 (0.03)defg
12 µM Kn	0.3	4.5 (0.41)bcd	0.39 (0.02)bcdefg
18 µM Kn	0.3	2.5 (0.29)e	0.29 (0.02)g

Table represents pooled means from 15 replicates in each of two repetitions; means followed by same letter(s) are not significantly different at alpha 5% level according to Tukey's test. Experiments were conducted using 0.75∇ MS medium, pH 5.8, 9% sucrose in the dark and data recorded after 60 days.

Effects of NAA : Effects of different concentrations of NAA were also investigated in a separate experiment and 0.3 µM NAA was found to be suitable obtaining to the highest number (8.7 ± 0.36) of bigger microrhizomes (0.82 ± 0.03), while both increasing and decreasing NAA concentration significantly decreased the number and size of microrhizomes (Fig. 3). Sunitibala et al. (2001) also observed that NAA (0.1 mg/l) is suitable for the induction of rhizomes. Peak and Murthy (2002) reported that NAA is the most effective auxin for the induction of bulblets *in vitro* from scale sections of *Fritillaria thunbergii*. Kim et al. (2003) also reported that 0.1 mg/l NAA together with 11% sucrose and 10 µM jasmonic acid showed the highest multiplication of bublets in garlic.

Effects of MS salts : Fig. 4 indicates the effects of MS salts on microrhizome induction in *C. longa*. Among the various strengths of MS salts, 0.75∇ strength of MS medium was found to be most suitable in terms of number (8.3 ± 0.55) and size

(0.81 ± 0.04 g) of microrhizomes, however, both half and full strength MS salts decreased the number (6.5 ± 0.53 and 7.1 ± 0.54) and the size (0.65 ± 0.04 g and 0.69 ± 0.04 g), respectively. A lesser strength of MS medium produced a lower number of smaller microrhizomes, while full strength MS produced a lower number but comparatively larger sized rhizomes. Shirgurkar et al. (2001) reported that half strength MS basal medium is suitable for microrhizome

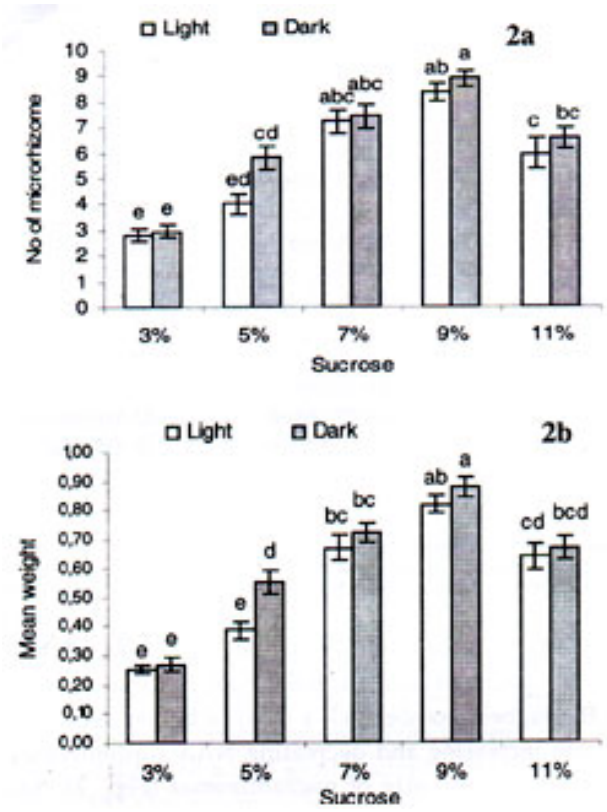

Fig. 2a, b. Effects of sucrose on *in vitro* microrhizome formation of *Curcuma longa* L. (experiments were conducted using 0.75 ∇ MS medium, pH 5.8, 6 µM BA, 0.3 µM NAA under 16 h light (L) and complete darkness (D) conditions). Results obtained from pooled means of two repeated experiments, each including 15 replicates; bars are standard error; means with the same letters are not significantly different at 5% level according to Tukey's test.

production in turmeric, obtaining an average number of 5.8 ± 0.7 with the biggest size being 0.55 ± 0.06 g, while Nayak (2000) and Sunitibala (2001) used full strength MS basal medium for microrhizome induction in *Curcuma*. Sharma and Singh (1995) also found full strength MS basal medium along with 7.5% sucrose and 35.2 µM BA optimal for the production of *in vitro* microrhizome in ginger.

Plantlet development and growth performance: Under *in vivo* conditions microrhizomes can successfully germinate after transferring them into soil (Fig. 1e). Germination, survival rate and morphological characters varied among the different sizes of microrhizomes. The survival rate of plantlets from

Table 2. Morphological parameters were evaluated using plants regenerated from different size of microrhizomes of *Curcuma longa* L. under *in vivo* condition.

Parameters	0.5 – 1.0 cm (SE)	1.1 – 2.0 cm (SE)	> 2.1 cm (SE)
Survival rate (%)	50	70	90
No. of shoots	0.6 (0.22)b	1.1 (0.28)b	2.3 (0.33)a
Length of shoots (cm)	6.8 (2.32)b	9.3 (2.07)ab	14.6 (1.76)a
No. of roots	3.9 (1.34)b	7.0 (1.63)ab	12.1 (1.46)a
Length of roots (cm)	5.4 (1.89)b	8.2 (1.94)ab	12.2 (1.50)a
No. of leaf	1.3 (0.47)b	2.7 (0.62)ab	3.3 (0.42)a
Leaf length (cm)	3.3 (1.12)b	5.6 (1.28)ab	10.7 (1.56)a
Leaf width (cm)	1.9 (0.66)a	3.6 (0.86)a	4.3 (0.58)a

Table represents pooled means from ten replicates. Means followed by same letters are not significantly different at alpha 5% level according to Tukey's test. Data recorded after 90 days cultivation in the glasshouse.

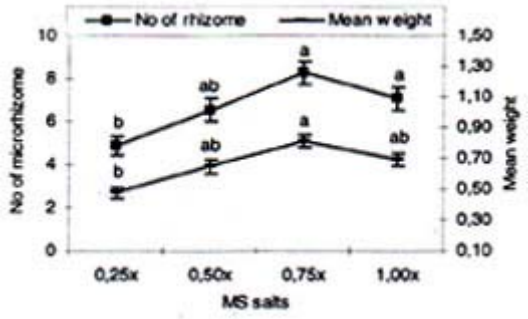

Fig. 3. Effects of NAA on *in vitro* microrhizome formation of *Curcuma longa* (experiments were conducted using 0.75∇ MS medium, pH 5.8, 12 µM BA and 9% sucrose in the dark). Results obtained from pooled means of two repeated experiments, each including 15 replicates; bars are standard error; means with the same letters are not significantly different at 5% level according to Tukey's test.

small (0.5 - 1.0 cm), medium (1.1 - 2.0 cm) and large (> 2.0 cm) microrhizomes was 50, 70 and 90%, respectively. Plants regenerated from bigger microrhizomes were also found to be more vigorous in the glasshouse in terms of their shoot, root and leaf growth parameters (Table 2). Similarly, Shirgurkar et al. (2001) reported that bigger microrhizomes were more competent and vigorous in the

field and grew faster. However, they obtained a survival rate of 10.4, 54.7 and 73.9%, respectively from smaller (0.1 - 0.4 g), medium (0.41 - 0.8 g) and large (> 0.8 g) microrhizomes. In our case, we obtained a much higher survival rate because the average weight of microrhizomes was higher, inferring that increasing production of larger microrhizomes is a key factor in achieving commercial success in microrhizome induction in *C. longa*. Our current protocol is one step towards achieving that commercial goal. Sharma and Singh (1995) reported that microrhizomes can be stored under moist conditions at room temperature and that more than 80% of the sprouted microrhizomes developed shoots and roots two months after they had been successfully transferred to the field.

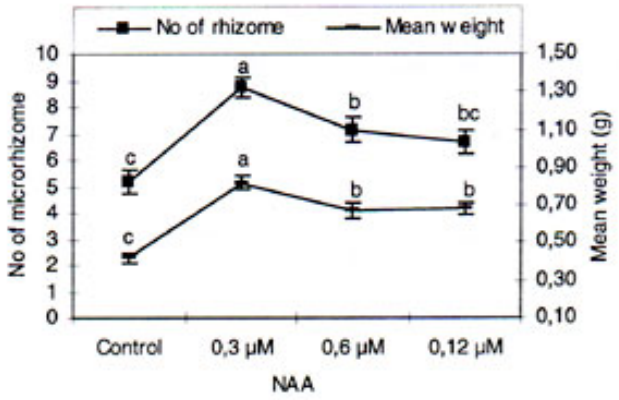

Fig. 4. Effects of the strength of MS salts on *in vitro* microrhizome formation of *C. longa* (experiments were conducted using 12 µM BA, 0.3 µM NAA and 9% sucrose in the dark). Results were obtained from pooled means of two repeated experiments, each including 15 replicates; bars are standard error; means with the same letters are not significantly different at 5% level according to Tukey's test.

In the present investigation the *in vitro* shoot multiplication system for *C. longa* has been optimised. Microrhizome induction in *Curcuma* needs further improvement. However, our developed protocol can be used to produce a higher amount of large microrhizomes compared to previously reported protocols. Production of *in vitro* microrhizomes would be a suitable source of disease free seed rhizomes that could be stored and transported easily. In addition to that, *in vitro* microrhizomes does not require acclimatization in the field. The present protocol is a step forward towards an improved commercial propagation system for *Curcuma longa* more efficient and productive than having a crop of plantlets.

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