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AN EFFICIENT METHOD OF MICROPROPAGATION OF GINGER (*ZINGIBER OFFICINALE* ROSC. CV. SUPRAVA AND SURUCHI) THROUGH *IN VITRO* RHIZOME BUD CULTURE

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SUMMARY

An efficient, simple micropropagation method was developed for *Zingiber officinale* Rosc. (cv, Suprava and Suruchi) using fresh rhizome sprouting bud in semisolid culture media. Explants were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations and combinations of BAP (6-benzyl- amino-purine) and NAA (α- naphthalene acetic acid) for shoot and root induction. Explants cultured on MS basal medium supplemented with 2.0 mg/l BAP + 0.5mg/l NAA showed highest rate of shoot multiplication. *In vitro* shoots were rooted on to the half strength MS basal medium supplemented with 2.0 mg/l NAA and rooting was better. Rooted shoots were transplanted in the green house for hardening and their survival was 95% in the field condition.

Key words: **Growth regulators, micropropagation, rhizome sprouting bud, tissue culture, *Zingiber officinale*.**

Zingiber officinale Rosc. (Ginger) of the family *Zingiberaceae* is an important tropical horticultural plant, values all over the world as an important spices for its medicinal properties. The *Zinggiberaceae* is an herbaceous moderate sized family of relatively advanced monocotyledonous plant of the order Zingiberales. Zingiberaceous plants are rhizomatous, perennial and aromatic herbs often of large size, bearing flowers either terminally on aerial leaf shoots or from ground level. These are plants of tropical and subtropical regions distributed mainly in East Asia. Several authors have quoted different figures for the total number of genera and species but it is probably appropriate to quote world record to be at least 51 genera and 1500 species (Newman 2001). The Indo-Malayan region is reportedly the centre of diversity for *Zingiberaceae* with at least 20 genera species occurring in Borneo, while Hottlum (1950) recorded 23 genera and 200 species. It is rich in secondary metabolites such as Oleoresin (Bhagyalakshmi and Singh 1988, Sakamura *et al*. 1986). Breeding of ginger is seriously handicapped by poor flowering and seed set. It is propagated vegetatively through rhizome. The germplasm collections in clonal repositories are also seriously affected by fungal diseases. Moreover since pathogenic fungi bacteria or viruses are readily transmitted through traditional practices, it was deemed important to develop a micropropagation technique and to make available for commercial use of pathogen free ginger germplasm. *In vitro* regeneration of auxiliary and adventitious shoots from shoot tips has already been established through callus culture by Hosoki and Sagawa (1977). Clonal multiplication methods through meristem tip culture have also been reported (Pillai and Kumar 1982, Smith and Hamill 1996). However in these methods, the propagation rate was not sufficient to obtain disease free quality microplantlets for commercial use and

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the acclimatization of the plantlet were very slow and unsatisfactory. Slow multiplication rate limited availability of high yielding genotypes and the extensive field maintenance of planting material, high susceptibility to rot diseases necessitates application of tissue culture techniques as a solution to these problems (Nayak and Nayak 2006). *In vitro* propagation has long been recognized as an efficient means for rapid clonal multiplication and conservation of important taxa. However *in vitro* culture is the best method as a continuous source of supply of disease free planting material for commercial utilization. Taking into account the utility, the various method of propagation including efficient cost effective method of *in vitro* multiplication is essential for improvement of ginger. The most important role of *in vitro* propagation is to conserve the genetic variation and evolutionary process in viable populations of ecologically and commercially viable varieties/ genotypes in order to prevent their potential extinction. In the present study we report rapid micropropagation of the two elite cultivar (cv. Suprava and Suruchi) which have high potential demand with good market value. The purpose of the study was to develop a technique for more rapid and more convenient clonal propagation of ginger in a cost effective manner for obtaining large scale disease free planting material for off season and year round cultivation for the benefit of the farmers by using fresh rhizome sprouting bud as an explant which was not included in the earlier studies.

MATERIALS AND METHODS

Explant source: The high yielding varieties (*Zingiber officinale* Rosc. cv- Suprava and Suruchi) of ginger were collected from High Altitude Research Station, Pattangi, Orissa University of Agriculture and Technology, Koraput, Orissa (Anonymous 2003). The rhizomes were planted in the nursery bed for sprouting. The young fresh buds of sprouting rhizomes were used for *in vitro* propagation. Healthy sprouts with active buds were collected from the rhizome of *Z. officinale* Rosc. (cv. Suprava and Suruchi) were maintained in the nursery bed of experimental garden of P.G. Department of Botany Utkal University. They were cut into 1.5 to 2.0 cm length with active buds intact. These rhizome sprouting with active buds were washed with 5% (v/v) detergent solution teepol (Qualigen, Mumbai, India) for 10 minute and rinsed several times with running tap water. These rhizome sprouting bud cuttings were surface sterilized with bavistin 0.3% followed by streptomycin 0.2 % for 10 minute and then washed with sterile distilled water. In the laminar chamber the sprouting bud cuttings were again treated with 70% alcohol for 30 second to one minute followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) treatment for 5 minute. Finally, the sprouting bud cuttings were washed thoroughly 3 to 4 times with sterile distilled water and blotted on paper and used as explants for raising *in vitro* cultures (Smith and Hamill 1996).

Culture medium and condition: The sterilized blotted explants were innoculated on to the Murashige and Skoog's (Murashige and Skoog 1962) agar medium fortified with various concentrations and combinations of growth hormones. For shoot induction, the medium was supplemented 0.25, 0.5, 1.0, 1.5, 2.0,2.5 and 3.0 mg/l BAP (6-benzyl- amino-purine) and 0.25 and 0.5 mg/l NAA (α-naphthalene acetic acid) either individually or in combination. For root induction *in vitro* raised shoots measuring about 4-5 cm grown in multiplication medium were cultured on half-strength MS medium supplemented with either NAA or IBA (indole 3-butyricacid) at concentration of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ l. The pH of the medium was adjusted to 5.8 before autoclaving at 1.04 kg/cm^2 pressure and 121°C temperature for 15 - 20 min. All cultures were maintained in 16 h light/8 h dark photoperiod (cool, white fluorescent light 30µmol $m^{-2}s^{-1}$). The cultures were incubated at 25 ± 3°C in light under 60 - 70% relative humidity in the culture room. The cultures were maintained by regular subcultures at 4 week interval on fresh medium with the same medium compositions.

Acclimatization: Rooted shoots were removed from the culture tube and the roots were washed under running tap water to remove agar .Then the plantlets were transferred to sterile poly pots (small plastic cups) containing vermiculite (TAMIN, India) and maintained inside growth chamber at 28^{0}C and 70-80% relative humidity. After three weeks, they were transplanted to earthen pots containing mixture of soil + sand + manure (FYM) in 1:1:1 ratio and kept under shade house for a period of three weeks for acclimatization.

Observation of cultures and presentation of results: Twenty culture tubes were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analyzed by the post-hoc multiple comparison test at the $P < 0.05$ level of significance (Marascuilo and McSweeney 1977).

RESULTS AND DISCUSSION

Shoot induction: The response of *Zingiber officinale* Rosc. (cv. Suprava and Suruchi) rhizome with slightly initiating buds as explants cultured on different shoot proliferation media over a period of six weeks is presented in Table1. Culture medium devoid of growth regulators (control) failed to stimulate the bud break

Table 1. Shoot formation in rhizome sprouting of *Zingiber officinale* Rosc. (cv. Suprava and Suruchi) cultured on semisolid MS medium supplemented with various concentration of BAP and NAA (20 culture tubes per treatment, data scored after 6 weeks.)

Different treatments	Hormonal supplements (mg/l)		% of Explant response	Days to bud break	Mean No of shoot/explant ±S.E.	Mean shoot length (cm) ±S.E.	Mean No of nodes/shoot ±S.E.
	BAP	NAA					
T1	0	0	-	-	-	-	-
T2	0.25	0	20	12-15	1.6 ± 0.30+	1.0 ± 0.09+	1.0 ± 0.12+
T3	0.5	0	30	12-15	2.5 ± 0.12+	1.5 ± 0.04+	1.5 ± 0.16+
T4	1.0	0	35	10-12	2.5 ± 0.38+	2.0 ± 0.14+	2.0 ± 0.18+
T5	1.5	0	40	10-12	2.6 ± 0.35	2.5 ± 0.26	2.0 ± 0.32
T6	2.0	0	50	8-10	2.8 ± 0.14	2.6 ± 0.23	2.2 ± 0.16
T7	2.5	0	45	8-10	2.0 ± 0.33+	2.8 ± 0.21+	2.2 ± 0.29+
T8	3.0	0	40	8-10	1.8 ± 0.49+	2.2 ± 0.18+	2.0 ± 0.33+
T9	0.25	0.25	30	10-12	2.1 ± 0.12+	2.4 ± 0.16+	1.5 ± 0.12+
T10	0.5	0.25	35	10-12	2.5 ± 0.14+	2.8 ± 0.04+	2.2 ± 0.16+
T11	1.0	0.25	40	7-9	3.0 ± 0.37+	3.0 ± 0.41+	2.0 ± 0.20+
T12	1.5	0.25	60	7-9	3.2 ± 0.25	3.0 ± 0.36	2.5 ± 0.12
T13	2.0	0.25	60	7-9	3.2 ± 0.33+	3.2 ±0.28	2.8 ± 0.09
T14	2.5	0.25	50	10-12	2 .2 ± 0.24+	2.0 ± 0.26+	2.5 ± 0.21+
T15	3.0	0.25	45	10-12	2 .0 ± 0.31+	1.8 ±0.12+	2.3 ±0.16+
T16	0.25	0.5	40	12-15	2.2 ± 0.28+	2.0 ± 0.28+	2.4 ± 0.16
T17	0.5	0.5	50	12-15	2.3 ± 0.04+	2.2± 0.30+	2.5 ± 0.08
T18	1.0	0.5	60	8-10	3.5 ± 0.42+	3.0 ± 0.32+	3.0 ± 0.20+
T19	1.5	0.5	75	6-8	4.5 ± 0.41	5.2 ± 0.43	4.7 ± 0.23
T20	2.0	0.5	90	6-8	7.5 ± 0.45	6.2 ± 0.37	5.6 ± 0.43
T21	2.5	0.5	60	8-10	3.5 ± 0.08+	3.2 ± 0.18+	2.8± 0.43+
T22	3.0	0.5	50	10-12	2.5 ± 0.43+	2.3±0.18+	2.0 ±0.14+

[20 culture tubes per treatment; repeated thrice. Means are calculated by post-hoc multiple comparisons tests at $P < 0.05$ level of significance ,+ callusing at the basal end , S.E.: Standard error of mean]

response in the explants even when the cultures were maintained beyond the normal observation period of four weeks. MS medium with growth regulator supplements produced better results in terms of percentage explants response, shoots /explant, average shoot length and average number of nodes produced per shoot. In such media combinations bud break was noticed within 6-8 days of culture (Fig.1a, Table.1**).** Of the combination tested MS+BAP (2.0 mg/l) + NAA (0.5 mg/l) elicited optimal response in which an average of 7.5 ± 0.45, shoots (Fig. 1a, Table.1) with a mean shoot length of 6.2 ± 0.37 cm per explant was recoded. The second best shoot multiplication (4.5 ± 0.41) shoot/culture was obtained in the medium MS+BAP (1.5 mg/l) + NAA (0.5 mg/l) with a mean shoot length of 5.2 ± 0.43cm. Higher concentration of BAP (2.5 & 3.0 mg/l) with NAA (0.5 mg/l) showed callusing of the explants with fewer number of shoots. In such cultures shoots were small with a mean shoot length of 2.5 ± 0.43 cm.

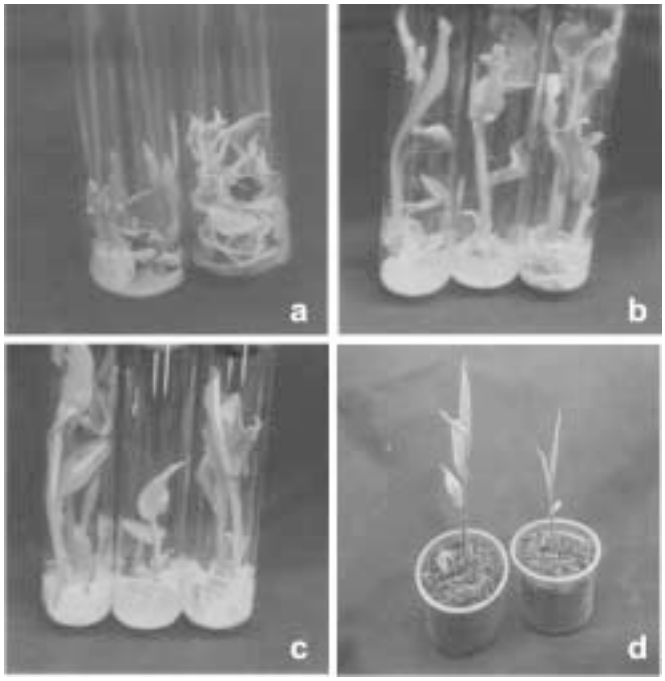

Fig. 1(a-d). *In vitro* regeneration and plant establishment of *Zingiber officinale* Rosc. (cv. Suprava and Suruchi (a) Multiple shoot emergence in MS+2.0 mg/lBAP + 0.5mg/l NAA, (b) Subculturing of the shoots in fresh shooting media, (c) Shoots rooted in 1/2MS+NAA (2.0 mg/l), (d) Hardening of rooted plantlets in plastic pots.

The dependence of cultured explants on bud break response and shoot multiplication has already been established and extensively discussed (Babu *et al.*1992). This has also been recently reported in the case of micropropagation of other *Zingiberaceae* like *Curcuma longa* (Balachandran *et al*.1990), *Z. officinale* (Bhat *et al*. 1994, Hosoki and Sagawa 1977, Sunitibala *et al*. 2001). In the present study, sprouting rhizome bud of *Z. officinale* Rosc.(cv. Suprava and Suruchi) showed significantly higher response in the medium with the combination of BAP (2.0 mg/l) + NAA (0.5 mg/l).The quality of shoots and the over all growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recorded when BAP was added alone in the medium. Review of literature indicates that the addition of either NAA or IBA or IAA in the culture medium improved the response in a number of species in terms of shoot growth. It has been reported that *Spathiphyllum floribundam* when cultured on media with BA supplement alone, a limited proliferation of explants with a maximum of average of 1.8 shoots per cultured explants was observed, while addition of IAA produced an average number of 11.6 shoots per explant (Malamug *et al*. 1991, Ramierez-Magon *et al*. 2001). Similar observation was reported in *Hovenia dulcis* nodal culture (Echeverrigaray *et al*. 1998). Rahman *et al*. (2004) reported that MS medium supplemented with 2.0 mg/l BAP is the best hormonal concentration for multiple shoot production in *Cucuma longa*. Similarly Panda *et al*. (2007) reported that, MS medium supplemented with 3.0 mg/l BAP is the best hormonal concentration for multiple shoot production in *Cucuma longa* (cv. Roma). In our study, it was observed that addition of NAA 0.5 mg/l with BAP (2.0mg/l) showed improved response over BAP alone. Some authors also suggested that the combination of BAP and NAA were needed for producing more number of multiple shoots on *Z. officinale* (Hashim *et al*. 1998, Hoque *et al*. 1999).

Rooting and establishment of plants in soil: The well developed elongated shoots measuring about 4-5 cm in length were excised from shoot clump and transferred to half-strength MS medium containing NAA or IBA. The rooting responses of excised shoots on different

media, which included rooting percentage, days required for root initiation, mean number of roots/shoot and mean root growth over a period of four weeks were different (Fig. 1b & c, Table 2).There was no rooting in case of shoot planted on auxin-free basal medium (control).Similarly, at lower level of NAA (0.25 mg/l) treatments, there was hardly any rooting in the cultured shoots during the 4 weeks of observation period. However higher concentration of NAA (1.5 & 2.0 mg/l) and IBA at all concentration tested responded well. Rooting was better in the culture which had combination of $^{1}/_{2}$ MS+2.0 mg/l NAA, where about 95% cultures responded with an average number of 8.5 ± 0.33 roots per plantlet and an average root length 3.5 ± 0.38 cm was recorded (Fig. 1b & c, Table 2) .The second highest response (70%) was recorded at 1.5mg/l of NAA with an average number of 6.0 ± 0.18 roots per plantlet and an average root length 3.0 ± 0.09 cm, which was similar with the previous report (Pillai and Kumar 1982, Sugaya 1991, Zakaria and Ibrahim 1986). In the present study, it was observed that root primordia emerged from the shoot base after 6 to 8 days of shoot transfer and soon after that the root growth was rapid. NAA has more effective than IBA in induction of rooting as days required to rooting was only 6-8 days as against 10 to15 days required for similar response in case of IBA.

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Sharma and Singh 1994, 1995).The auxins NAA and IBA were used singly to induce rooting from *in vitro* raised shoots. Different concentration of auxin (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) were tried for development of root system. In our study half-strength MS basal medium supplemented with different concentration of NAA and IBA exhibited optimum

Table 2. Influence of different levels of NAA and IBA on rooting response of *in vitro* generated shootlets of *Zingiber officinale* Rosc. (cv. Suprava and Suruchi) (20 culture tubes per treatment, data scored after 4 weeks.)

Different treatments	Growth regulators augmented with1/2 strength MS basal medium(mg/l)		% of Explant Response	Days to root initiation	Mean root numbers ±S.E.	Mean root length (cm) ±S.E.
	NAA	IBA				
T1	0	0	-	-	-	-
T2	0.25	0	20	12-15	1.0 ±0.30	1.0 ± 0.14
T3	0.5	0	40	10-12	2.0 ± 0.24	2.0 ± 0.12
T4	1.0	0	50	10-12	2.5 ± 0.04	2.2 ± 0.16
T5	1.5	0	70	6-8	6.0 ± 0.18	3.0 ± 0.09
T6	2.0	0	95	6-8	8.5 ± 0.33	3.5 ± 0.38
T7	2.5	0	50	10-12	2.2 ±0.32+	2.3 ±0.14+
T8	3.0	0	40	10-12	1.4 ±0.24+	1.7 ± 0.28+
T9	0	0.25	30	12-15	1.0 ± 0.14	1.5 ± 0.12
T10	0	0.50	40	12-15	2.2 ± 0.16	2.4 ± 0.16
T11	0	1.0	60	10-12	2.5 ± 0.04	2.5 ± 0.12
T12	0	1.5	55	10-12	2.6 ± 0.04	2.2 ± 0.08
T13	0	2.0	70	10-12	2.8 ± 0.12	2.6 ± 0.04
T14	0	2.5	50	10-15	1.8 ±0.38+	2.2 ±0.14+
T15	0	3.0	40	10-15	1.6 ±0.14+	1.5 ±0.14+

[20 culture tubes per treatment; repeated thrice. Means are calculated by post-hoc multiple comparisons tests at $P < 0.05$ level of significance ,+ callusing at the basal end , S.E.: Standard error of mean]

growth pattern. The maximum results on rooting were obtained on half-strength with NAA (2.0 mg/l) than IBA (2.0 mg/l). Rahman *et al*. (2004) reported that half-strength MS medium supplemented with 0.1-1.0 mg/l of any auxin (NAA, IAA and IBA) was effective for rooting in *Curcuma longa* (found in Bangledsh). Our observations are in accordance with the result of Inden *et al*. (1998) in *Z. officinale*.

The well rooted plants were transferred to sterile poly pots (small plastic cups) containing pre-soaked vermiculite (TAMIN, India) and maintained inside growth chamber at 28^{0}C and 70-80% relative humidity. After three weeks they were transplanted to earthen pots containing mixture of soil + sand + manure (FYM) in 1:1:1 ratio and kept under shade house for a period of three weeks for acclimatization. (Fig. 1d).Upon transferred to shade house, plants started producing fresh shoots after two weeks of trans- planting. Later they were transferred to the field condition and the survival rate was 95%. The efficient micropropagation technique described here may be useful for raising disease-free quality planting propagules of *Z. officinale* for commercial and off season cultivation which not only help the socioeconomic development of the farmers but also fulfill the spice value and market demand (Kuruviashetty *et al*.1982, Noguchi and Yamakawa 1998).

The fresh rhizome sprouting explants in both the cultivated variety of *Z. officinale* Rosc. (cv. Suprava and Suruchi) respond well and is the appropriate explants for the formation of multiple shoots in MS medium supplemented with BAP (2.0 mg/l) + NAA (0.5 mg/l), where as $^1/_2$ MS+2.0 mg/l NAA is the standard growth regulators for profuse rooting. Both the varieties grow well in the said culture medium perhaps due to close genometic loci among themselves even possessing some morphotypic variation.

In conclusion, the protocol with a high rate of multiplication can be successfully utilized for commercial scale of disease free, quality plantlet production including conservation and off season year round cultivation of both the variety for socioeconomic development of the farmers.

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