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MICROPROPAGATION OF GINGER *(ZINGIBER OFFICINALE* VAR. RUBRUM) USING BUDS FROM MICROSHOOTS

ZURAIDA A.R.[1*], MOHD SHUKRI M.A.[2], ERNY SABRINA M.N.[3], AYU NAZREENA O[1], CHE RADZIAH C. Z.[4], PAVALLEKOODI G.[5] AND SREERAMANAN S.[5]

[1]*Biotechnology Research Centre, MARDI Headquarters, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia*
[2]*Genebank and seed centre, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia*
[3]*Agrobiodiversity & Environment Research Centre, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia*
[4]*Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia.*
[5]*School of Biological Sciences, Universiti Sains Malaysia, Georgetown, 11800, Penang, Malaysia*
[*]*Corresponding authors' e-mail: azuraida@mardi.gov.my*

Abstract

Zingiber officinale var. *Rubrum* (ZOR) is cultivated for its medicinal value despite the constraints of longer life cycle. The study has established an efficient and reproducible protocol to micropropagate ZOR using buds generated on the surface of the ginger. Surface sterilized young buds of 0.5-1 cm and 2-4 cm cultured on Murashige and Skoog (MS) supplemented with BAP showed the highest survival rate (55-65%) and produced the highest average number of microshoots per explant (3.2±0.06) respectively. MS medium supplemented with different concentrations and combinations of auxin and cytokinin were used to evaluate shoot multiplication and root induction. BAP concentrations between 3.0-5.0 mg/L was very effective in promoting microshoots and resulted in 100% of microshoot propagation. Microshoots cultured on MS medium supplemented with 3 mg/L BAP and 0.5 mg/L NAA produced the highest number of shoots while 0-0.5 mg/L BAP enhanced shoot length and 3mg/L NAA in combination with BAP produced highest number of roots. Microshoots maintained on MS medium supplemented with 4.5% sucrose produced the highest number of plantlets (23±2.5) and roots per explants (15.4±2.4) meanwhile reducing the length of lateral roots (2.6±0.2).

Key words: *Zingiber officinale*, Micropropagation, Plant growth regulator, Carbon source, Young bud.

Introduction

Ginger (*Zingiber officinale*) is a monocot plant belongs to the family *Zingiberaceae*. Ginger is planted in the tropics for its edible rhizomes serving culinary and medicinal purposes (Portnoi *et al*., 2003). Rhizome part of the ginger plant is commonly used as condiments in food preparation to contribute to the taste and flavour (Larsen *et al*., 1999). Apart from the flavor, ginger is loaded with bioactive phytochemicals, mainly gingerols, shagols as well as volatile oils such as sesquiterpenes (β-bisabolene and (−)-zingiberene) and monoterpenes (geranial and neral) (Ursell, 2000; Chevallier, 1996). The rhizome bark was reported to accumulate anthocyanin and tannin (Li *et al*., 2001).

Two varieties of ginger: *Zingiber officinale* var. roscoe (white ginger) and *Zingiber officinale* var. rubrum (red ginger) have been indentified in Malaysia and neighbouring South-east Asian countries (Weiss, 2002). *Z. officinale* var. rubrum is locally known as Jahe Merah or Halia Bara in Indonesia and Malaysia, respectively. The local names defined as red ginger referring to the reddish-purple surface of the rhizome (Ravindran & Babu, 2005) partly due to the anthocyanin accumulation. The reddish rhizome however comes in a yellow to pinkish cross-section which is smaller in size and very pungent (Weiss, 2002 ; Ibrahim *et al*., 2008). As mentioned previously, *Z. officinale* var. rubrum is a pharmaceutically important herb to treat rheumatism, osteoporosis, asthma, cough, stomach discomfort, tumours, and as a postpartum medicine (Ibrahim *et al*., 2008 ; Wan Ibrahim *et al*., 2010).

ZOR is commercially cultivated in tropical and south-east Asia regions such as Malaysia, Indonesia, India, Thailand and China. Furthermore, it is an annual crop with less care with high economic return. Generally underground rhizomes are used as the conventional planting materials. However, the conventional method is accompanied by low multiplication rate. Furthermore, ginger plants are prone to fungal, bacterial, viral and mycoplasma diseases. For example, *Pythium aphanidematum* causing soft rot, *Fusarium oxysporum* causing yellowing of leaf, *Pseudomonas solanancearum* causing bacterial wilt, *Phyllosticra zingiberi* causing leaf spot in addition to shoot borer *Conogethes punctiferalis* and and root-knot nematode *Meloidogyne incognita* leading to crop losses (Kavyashree, 2009). Besides, maintenance of germplasm by annual plantation is expensive and labouris (Balachandran *et al*., 1990). Tissue culture is the only methodology that can produce a large quantity of clonal plants in a short time with high phytosanitary quality (Silva *et al*., 2014), therefore, it is important to generate disease free clones in large numbers in short time and space via *In vitro* plant tissue culture technique to ensure a continuous supply of ginger to the farmers and consumers.

Thus, development of reproducible *In vitro* protocols to allow rapid micropropagation of ginger via direct organogenesis or somatic embryogenesis became necessary. Various explants such as vegetative buds (Sharma & Singh, 1997 ; Kavyashree, 2009) and shoot tips (Malamug *et al*., 1991) have been used as explants to establish *In vitro* cultures of ginger. The present study describes an efficient protocol for the micropropagation of ZOR.

Materials and Methods

Establishment of initial cultures: Healthy *Zingiber officinale* var. rubrum (ZOR) plants were maintained in glass house of Malaysian Agricultural Research and Development Institute (MARDI) for few weeks to allow sprouting of young buds. Young buds at different size ranging from 0.5 to 5.0 cm were collected and used as the source of explants. The young bud were then washed under running tap water containing commercial laboratory detergent (Decon 5%, v/v) for an hour and rinsed thoroughly with water. The explants were brought inside laminar air flow chamber to surface sterilize with 20% of Clorox® (5.25% NaOCL) and 50 uL (micro liter) of Tween-20 for 30-40 mins on a rotary shaker. The explants were then rinsed several times with sterile distilled water. The outer layers of leaf sheaths of sterilized explants were removed until 5 to 8 mm sized meristem pieces having at least one eye is produced. The explants were then cultured onto solidified Murashige and Skoog's (MS) (1962) basal medium supplemented with 3% sucrose with three different concentration of cytokinin 6-benzylaminopurine (BAP) (0, 1.0 and 5.0 mg/L). The pH of the medium was adjusted to 5.8 prior to autoclaving (15 min, 121°C). The cultures were incubated in the culture room under white fluorescent light with light intensity of 50 µM $m^{-2}s^{-1}$ at a photoperiod of 16h at 25±2°C. Cultures were checked regularly for contaminations and those presented apparent infection symptoms were immediately discarded. Data were expressed as means days to first shoot appearance, percentage of survival after 45 days of culture and average number of microshoot per explant.

Multiplication: Clump of microshoots with basal part attached maintained on MS basal with 1 mg/L BAP was used as explants source in this experiment. The microshoots were cultured on MS basal containing 3.0% sucrose and 0.3% Gelrite. The media was supplemented individually with five different concentrations of the BAP (0, 0.5, 1.0, 3.0, 5.0 and 7.0 mg/L) or kinetin (Kin) (0, 0.5, 1.0, 3.0, 5.0 and 7.0 mg/L) for propagation of microshoots. Observations were recorded at weekly intervals. Results were expressed as explants forming new shoots (%) and number of microshoots/explants.

The effect of cytokinin (BAP) and auxin (NAA) on shoot multiplication was examined in a separate experiment. Microshoots were transferred onto MS medium supplemented with various concentration of BAP (0, 0.5, 1.0, 3.0, 5.0 mg/L) alone or BAP (1.0, 3.0, 5.0 mg/L) in combination with NAA (0.5 and 3.0 mg/L). Mean number of shoots, shoot length and number of roots were recorded after 45 days of culture.

Effect of sucrose and acclimatization: In order to enhance the shoot multiplication, explants were subcultured onto medium containing 3 mg/L BAP and 0.5 mg/L NAA. The medium comprised of five sucrose concentrations (10, 15, 30, 45 and 60 g/L). The cultures were incubated in the culture room under white fluorescent light with light intensity of 50 µM $m^{-2}s^{-1}$ at a photoperiod of 16h at 25±2°C. Plantlets were harvested after 30 days and the number of complete plantlets (i.e. plantlets with roots), number of root and average length of roots were recorded.

Plantlets with roots were then removed from the culture bottles and the roots were washed under running tap water to remove the agar. The plantlets were then individually transplanted into polybag (containing organic soil and topsoil at the ratio of 1:1) and kept in glasshouse under 75% shading. The plantlets were watered periodically. The survival rate of the plantlets was recorded after 6-8 weeks.

Results and Discussion

Effect of various sizes of explants on mean days for first bud appearance, percentage of survival rate, and mean number of microshoot: Contamination and low multiplication rates are the limiting factors of generating healthy and uniform clones of an *In vitro* plantlet (El Boullani *et al*., 2013). Moreover, Barcelo-Munoz *et al*. (1999) reported that juvenile explants may reduce the occurrence of necrosis and hyperhydricity which is the main concern when using mature tissues to induce *In vitro* culture. Hence, the study was carried out using young buds. Young buds of ZOR at various sizes (0.5-1, 2-4, more than 4cm) were treated with various BAP concentration (0, 1, and 5 mg/L) to study the days taken for first bud appearance, survival of *In vitro* cultures after 45 days, and number of microshoot per explants. Based on the results, explants of 0.5 to 1cm took longer days for first bud appearance and scored highest percentage of survival after 45 days. Contrarily, explants of 2 to 4 cm and over 4cm took lesser days in the range of 31 to 35 days for first bud appearance and their survival after 45 days significantly decreased to the range of 25 to 35 days. Furthermore, browning of the tissues was predominant on the over 4cm explants. Browning is an indication of phenolic oxidation. Leaching of phenolic compounds during explants excision readily oxidizes and become phytotoxic to cause necrosis and death (Preece & Compton, 1991). Thus, browning of the bigger sized explants is correlated to the lower survival percentage. Normally, bigger explants produce more amount of ethylene than the lower explants, consequently ethylene influences in the explant oxidation (Silva *et al*., 2015), and higher ethylene levels can induce explant death.

However, explants of 2-4 cm produced the highest number of microshoot per explants; scoring 2.2 ± 0.31 and 3.2 ± 0.06 at 1 and 5 mg/L BAP respectively (Table 1). Nasirujjaman *et al*. (2005) and Kambaska & Santilata (2009) have documented the beneficial role of BAP in shoot multiplication of *Zingiberaceae* species such as turmeric and ginger, respectively. The highest number of microshoot was recorded (3.2 ± 0.06) on 2-4 cm sized explants treated using MS supplemented with 5 mg/L BAP. This result was followed by the similar explants treated with 1 mg/L BAP. Contrarily, Kiranmayee *et al*. (2011) recorded highest (11.66□±□1.15) shoot induction on *Alpinia galangal* rhizome cultured on MS supplemented with 2 mg/L BAP. However, higher concentrations of cytokinin may reduce the number of shoots due to the hyperhydricity effect (Paek & Hahn, 2000; Armstrong & Johnson, 2001; Oliveira *et al*., 2010).

Table 1. Effect of various sizes of explants on mean days for first bud appearance, percentage of survival rate, and mean number of microshoots.

Size of explants (cm)	BAP (mg/L)	Mean days for first bud appearance	Survival after 45 days (%)	Mean number of microshoot per explants
0.5-1	0	35	65 ± 6.9	0.7 ± 0.01
	1	39	55 ± 5.5	0.5 ± 0.01
	5	40	55 ± 5.7	1.1 ± 0.12
2-4	0	32	35 ± 2.4	0.6 ± 0.01
	1	35	35 ± 4.5	2.2 ± 0.31
	5	35	25 ± 1.3	3.2 ± 0.06
>4	0	31	35 ± 4.1	0.4 ± 0.02
	1	33	30 ± 1.1	0.9 ± 0.09
	5	33	25 ± 2.8	1.2 ± 0.55

Effect of plant growth regulators on multiplication of *Z. officinale* var. rubrum: In the preceding experiment, the analysis on the effect of cytokinin on the development of microshoots on the explants was limited to BAP at 0, 1, and 5 mg/L and it was proven that cytokinin influence the development of microshoots on the explants. Hence, an in depth experiment was then conducted using BAP and kinetin individually at 0, 0.5, 1, 3, 5, and 7 mg/L to obtain optimal response. Microshoots with basal part (length about 0.5-1.0 cm) were used as the explants to carry out the study for duration of 45 days. Based on the results, 3 and 5 mg/L BAP produced the highest number of microshoots (7±1.34 and 6±0.41, respectively) as proven in the previous experiment. Furthermore, 3 and 5 mg/L BAP promoted 100% of microshoots. Fig. 1a and b depict the regenerated microshoots. However, increasing the concentration of BAP further to 7 mg/L decreased both the number of microshoots. Although 0.5 and 1.0 mg/L BAP produced same number of microshoots; the later increased the microshoot percentage significantly from 55±6 to 70±11 %. Generally, a pattern of significant increment in the percentage of microshoots was observed as the concentration of BAP increased compared to control.

A similar normalized bell shape pattern was observed for the effect of kinetin on percentage of microshoots developed on microshoots with basal part. 3 mg/L kinetin significantly produced multiple microshoots (4±0.88) and enhanced subsequent multiplication (45±6). Contrarily, Lindiro *et al*. (2013) reported that nodal explants of *Chrysanthemum cinerariaefolium* maintained in MS medium supplemented with 40 µM kinetin gave the highest mean number (14.33±0.66) of microshoots per explant and the highest mean length (1.12±0.04) of shoots; which was comparable to that of the best BAP concentration tested. According to Table 2, kinetin at all the other tested concentrations produced poor response in terms of development of microshoots and propagation of microshoots. For example, too low (1 mg/L) or too high (7 mg/L) of kinetin concentration caused development of single microshoot per explant. Furthermore, it can be concluded, increasing kinetin above 5 mg/L may give poorer results compared to the control. On the other hand, Walia *et al*. (2007) reported that Kinetin alone at 2.22 µM produced single microshoot per nodal explants (sixth to ninth from the shoot tip). However, a combination of 2.22 µM BAP and 2.22 µM kinetin enhanced multiple microshoot on nodal explants due to the interactive effect of the two cytokinins (Walia *et al*., 2007). Similarly, Chirangini & Sharma (2005) observed 80% of the rhizomatous eye/bud maintained in MS medium supplemented with 2.32 µM kinetin produced single microshoots.

Effect of plant growth regulators on mean number of shoots, shoot length and mean number of roots of *Z. officinale* var. rubrum. The positively stimulating effect of BAP on *Z. officinale* var. *rubrum* was further investigated in terms of shoot length and number of roots together with number of shoots. The parameters were analyzed on the microshoots (initiated on MS supplemented with 3.0 mg/L BAP) maintained on MS supplemented with BAP alone (0, 0.5, 1, 3, 5 mg/L) or BAP (1, 3, 5 mg/L) in combination with NAA (0.5 or 3 mg/L) for 30 days. The interactive effect between BAP (1, 3, 5 mg/L) and NAA (0.5 mg/L) produced the highest number of shoots (in the range of 14-19 shoots per explants) although BAP alone at 1 and 3 mg/L produced 12-13 shoots and 9 roots per explants simultaneously. For example, culture jar in which explant grown on MS supplemented with 3 mg/L BAP and 0.5 mg/L NAA is dense with multiple shoots (Fig. 1c).

Similarly, shoot buds of *Boesenbergia rotunda* (L.) Mansf. cultured on MS medium supplemented with 2 mg/L BAP and 0.5 mg/L NAA produced maximum number of multiple shoots (5). However, this Zingiberaceae member produced only 1 to 2 shoots per explants and abnormal in morphology when challenged with NAA alone or increasing above 1 mg/L or BAP above 3 mg/L (Yusuf *et al*., 2011). Furthermore, Balachandran *et al*. (1990) reported that MS medium supplemented with 3 mg/L BAP was optimal for rhizome buds of turmeric (*Curcuma domestica C. caesia* and *C. aeruginosa*) and ginger (*Zingiber officinale* Rosc) to produce multiple shoots. In accordance to the current study, shoot tip of *Curcuma longa* L. cultured on MS supplemented with 2 or 3 mg/L BAP and 1 mg/L NAA produced highest average number of shoot (2.4 and 2.6 shoots, respectively) and optimal number of root (3 and 2.6 roots, respectively) (Jala, 2012). Table 3 shows that absence of NAA or increasing to 3 mg/L in combination with BAP does not imply any significant effects on the number or length of shoots except the latter increased the number of roots (in the range of 10-11 roots per explants). The synergistic effect of BAP (3 and 5 mg/L) and NAA (0.5 mg/L) on *In vitro* shoots of *C. mangga* to form root (9.8±1.4 and 11.2±2.3) has been reported byRaihana *et al*. (2011).

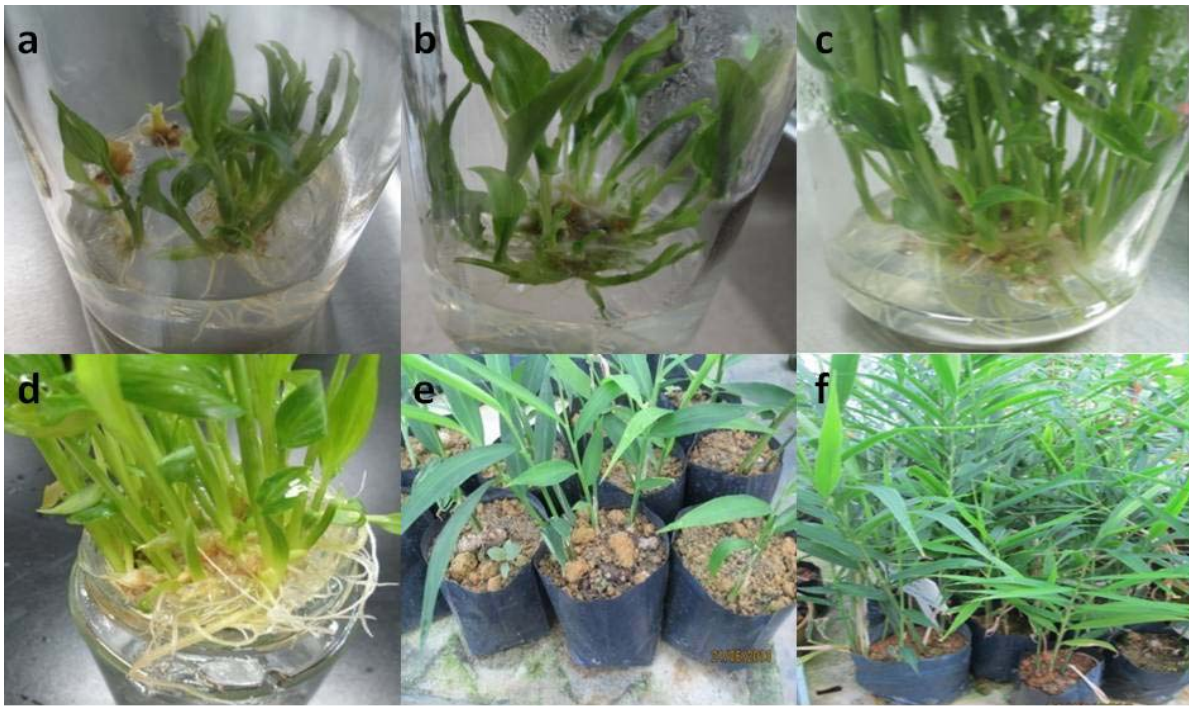

Fig. 1. Regeneration of *In vitro* plantlets of *Zingiber officinale* var. rubrum. Regenerated microshoots (a-b), plantlets grown in MS supplemented with 3 mg/L BAP and 0.5 mg/L NAA (c), rooted plantlets grown in MS supplemented with 4.5% sucrose (d), 6-8 weeks old plantlets kept at the glasshouse under 75% shading (e-f).

Table 2. Effect of plant growth regulators on multiplication of microshoots on *Z. officinale* var. rubrum.

BAP (mg/L)	Kin (mg/L)	Explants forming new microshoots (%)	Number of microshoots/ explant
0	0	45±5	3±0.43
0.5	0	55±6	4±0.56
1.0	0	70±11	4±0.11
3.0	0	100±13	7±1.34
5.0	0	100±17	6±0.41
7.0	0	60±8	2±0.07
0	0.5	20±3	2±0.06
0	1.0	25±5	1±0.2
0	3.0	45±6	4±0.88
0	5.0	25±2	3±0.31
0	7.0	10±2	1±0.12

Table 3. Effect of plant growth regulators on mean number of shoots, shoot length and mean number of roots of *Z. officinale* var. rubrum.

BAP (mg/L)	NAA (mg/L)	Mean number of shoots	Shoot length (cm)	Mean number of roots
0	0	5 ± 0.93	6-7	5.9 ± 2.13
0.5	0	9.3 ± 1.34	6-7	7.0 ± 3.31
1.0	0	12.0 ± 2.11	4-5	9.1 ± 3.40
3.0	0	13.6 ± 3.14	3-5	9.1 ± 1.20
5.0	0	9.5 ± 0.67	3-5	8.± 50.91
1.0	0.5	16.4 ± 2.1	4-5	8.9 ± 1.4
3.0	0.5	19.5 ± 2.3	4-5	7.3 ± 2.1
5.0	0.5	14.5 ± 3.1	3-5	8.3 ± 0.8
1.0	3.0	11.4 ± 11.1	4-5	11.6 ± 2.1
3.0	3.0	11.1 ± 0.9	3-5	11.1 ± 1.1
5.0	3.0	9.3 ± 1.4	3-5	10.4 ± 0.9

Table 4. Effect of different sucrose concentration on propagation and rooting of *Z. officinale* var. rubrum cultured on MS medium supplemented with 3 mg/L BAP and 0.5 mg/L NAA.

Sucrose concentration (g/L)	Number of plantlets	Number of root per explants	Average length of roots (cm)
10	10 ± 0.6	7.2 ± 0.51	5.52 ± 0.5
15	13 ± 1.5	9.1 ± 1.3	6.3 ± 0.7
30	17 ± 3.5	8.9 ± 2.1	3.4 ± 0.5
45	23 ± 2.5	15.4 ± 2.4	2.6 ± 0.2
60	19 ± 3.5	13.5 ± 0.8	2.1 ± 0.3

Furthermore, it is evident that plant growth regulators is not needed to enhance the shoot length of ZOR since the control recorded the highest shoot length (Table 3). Similarly, Ayadi *et al*. (2011) observed MS medium supplemented with 2mg/L BAP reduced the shoot length of kenaf (*Hibiscus cannabinus*) seedlings to 2.3cm compared to MS control medium that enhanced the shoot length to 8 cm. Furthermore, higher BAP concentrations were suggested to reduce shoot length, number of shoots and to cause abnormalities on *In vitro plant* cultures (Jafari *et al*., 2011; Sathyagowri & Seran, 2011).

Effect of different sucrose concentration on *In vitro* rooting of *Z. officinale* var. rubrum cultured on MS medium supplemented with 3 mg/L BAP and 0.5 mg/L NAA: Apart from phytohormones, carbon source is also an important factor to be considered for the *In vitro* propagation of plants. Generally, sucrose is used to provide carbon source and energy and to maintain a stable osmotic pressure environment to sustain the growth of *In vitro* cultures of plants (Chen *et al*., 2007; Das *et al*., 2010).

Hence, MS medium was supplemented with 3 mg/L BAP, 0.5 mg/L NAA and sucrose at various concentrations (10, 15, 30, 45, 60 g/L) to study the effect of it on rooting rate of *Z. officinale var. Rubrum* after 45 days. Table 4 shows that increasing sucrose concentration increases number of plantlets and number of roots per explants but decreases the average length of roots. However, the highest tested concentration of sucrose (60g/L) caused reduction in the number of plantlets and roots. On the other hand, Zheng *et al*. (2008) reported that 80 g/L sucrose was ideal for *In vitro* microrhizome production of *Z. Officinale* Roscoe. Based on the results sucrose at 45 g/L is the optimal concentration since the treated explants produced the highest number of plantlets (23±2.5 cm) and roots (15.4±2.4 cm). Fig. 1d depicts dense roots formed on the explant maintained in MS supplemented with 45 g/L sucrose. However, longer roots requires lesser sugar concentration because optimal concentration of sugar halved the length of lateral roots (2.6±0.2) compared to 1 and 15 g/L sucrose treated explants (5.52±0.5 and 6.3±0.7 cm respectively). Contrarily, Das *et al*. (2012) reported that buds of *Z. moran* and *Z. zerumbet* maintained on MSR medium supplemented with 20 g/L sucrose showed greater shoot multiplication while concentrations higher than 30 g/L caused a defoliating effect on the both species. However, MS medium supplemented with 30 g/L sucrose with has been used for micropropagation of members of Zingiberaceae (Tyagi *et al*., 2004; Stanly & Keng, 2007).

Sucrose treated plantlets that trasnferred to polybags and maintained at greenhouse were analysed for the survival rate. Hundred (100) plantlets that were used to study survival rate after 6-8 weeks were remained healty and regenarated to complete ZOR plants (Fig. 1e and f) ensuring 100% survival rate. Hence, it can be concluded that ZOR plantlets produced through plant tissue culture technique were free from contamination and morphologically identical to the wild ZOR plants.

Conclusion

This study established a rapid micropropagation method for ZOR involving plant growth regulators. BAP in combination with lower concentration of NAA synergistically enhanced number of shoots but antagonistically reduced the shoot length while increased the number of root in combination with higher concentration of NAA. Plantlet and root formations were also parallel to the addition of sucrose suggesting a synergistic effect. The development of the micropropagation protocol for genetically uniform ZOR plants will meet the demand for this plant in pharmaceutical field, curb overexploitation of the herb for traditional medicine, allows systematic cultivation and germplasm conservation.

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