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Full Length Research Paper

In vitro propagation of Ethiopian ginger (*Zingiber officinale* Rosc.) cultivars: Evaluation of explant types and hormone combinations

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Ginger (*Zingiber officinale* Rosc.) is the second most widely cultivated spice in Ethiopia, next to chilies. Recently, there has been huge demand for clean planting material of improved ginger cultivars, though it is difficult to meet the demand of planting materials using the conventional propagation techniques due to production inefficiency and disease transmission. Therefore, the present study was carried out with the objective of assessing the potential of axillary buds and shoot tips as explant sources and determination of suitable growth regulators for *in vitro* propagation of two ginger cultivars. Murashige and Skoog (MS) medium with four levels of benzyl adenine (BA) and kinetin was used for shoot multiplication in combination with two explant sources. A highly significant difference ($p<0.0001$) was observed between explant sources and among growth regulators for shoot multiplication. From this study, it was found that shoot tip explants on 2 mgl^{-1} BA and 1 mgl^{-1} kinetin was found to be better than other explant-media combinations which gave an average of 7 shoots per explant within six weeks of culture. Consecutively, the plantlets developed an average of 8.75 roots within four weeks of culture period and performed well in acclimatization and subsequently in the field.

Key words: Ginger, *Zingiber officinale* Rosc., growth regulators, explants, micropropagation.

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), a member of the family Zingeberaceae, is an important tropical herbaceous perennial plant, with the rhizome valued for its culinary and medicinal properties. Ginger production for the extraction of oleoresins and essential oils, as well as the direct use of rhizomes for culinary purposes is increasing worldwide (FAO, 2008). In 2007, it was the second widely cultivated spice (224,580 tons from 17,550 ha of land) next to chilies in Ethiopia (Girma et al., 2008; MoARD, 2008).

Although, there are more than 45 ginger cultivars in the country (MoARD, 2008), their production and productivity is low. Among the major production problems of ginger in Ethiopia, crucial shortage of planting material is one of the major bottlenecks (MoARD, 2008). Conventionally, propagation of ginger depends solely on the use of smaller rhizome pieces or setts (Narong, 1996) with about 2.5 ton setts requirement to cover a hectare of land (1,000 kg/acre) (UEPB, 2005). This is so bulky and difficult to obtain when one require establishing a new farm or expanding its area of cultivation. The problem gets aggravated particularly in cases where producers wish to replace their old clones with newly developed elite materials. Therefore, the use of *in vitro* propagation techniques becomes imperative to alleviate the shortages of planting material.

Earlier, Hosoki and Sagawa (1977) were the first to report their success in the production of an average of 6 shoots per bud from *in vitro* culture, though the field survival rates recorded were low. From that time onwards, several workers had succeeded in their *in vitro*

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Table 1. Treatment arrangements of multiplication and rooting experiments.

Treatment																		
	Multiplication experiment	BA (mg $^{-1}$)	0	0	0	0	1	1	1	1	2	2	2	2	3	3	3	3
		Kinetin (mg $^{-1}$)	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
	Rooting experiment	BA (mg $^{-1}$)	0	0	0	0	2	2	2	2	4	4	4	4	6	6	6	6
		NAA (mg $^{-1}$)	0	0.5	1	1.5	0	0.5	1	1.5	0	0.5	1	1.5	0	0.5	1	1.5
Treatments for the experiments			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

culture of ginger following the organogenesis pathway (Balachandran et al., 1990; Dekkers et al., 1991; Villamor, 2010), while others had used the indirect somatic embryogenesis pathway (Malamug et al., 1991; Babu et al., 1992; Kackar et al., 1993; Azra et al., 2009). On the other hand, Sharma et al. (1995) had successfully produced disease free ginger micro rhizomes *in vitro*. Others (Dekkers et al., 1991) have also used successfully the *in vitro* technique for conservation of germplasm.

Recent works on ginger by Villamor (2010) and Azra et al. (2009) showed an average of 6.6 and 3.8 shoots through organogenesis and indirect somatic embryogenesis, respectively which is in efficient for mass propagation. Besides, *in vitro* propagation requires optimization of appropriate protocols for new cultivars. However, no such work had been carried out in Ethiopia to optimize the *in vitro* mass propagation protocol for newly released elite ginger varieties. Therefore, it is necessary to optimize the tissue culture propagation protocol for these two varieties to rapidly propagate and disseminate the materials to producers across the ginger growing belt of the country. Thus, this research work was conducted with the objective of assessing the potential of axillary buds and shoot tips as explant sources for shoot multiplication through optimization of the plant growth regulators.

MATERIALS AND METHODS

Genetic materials

Two recently released elite Ethiopian ginger cultivars, Yali (180/73) and Boziab (37/79) obtained from Teppi Agricultural Research Center were used in this study. The two cultivars have high essential oil and oleoresin oil content and a yield potential of 200 to 250 qt ha^{-1} on average.

Preparation of explants

The selected healthy and uninjured fresh rhizomes were thoroughly washed with common liquid soap and retained in a moist bed laid with absorbent cotton in store house with room temperature of 20 to 25°C. The rhizomes were watered regularly using distilled water for 15 to 20 days to initiate sprouting. Sprouted shoot tips and axillary buds from these rhizomes were used as a source of explants for subsequent experiments.

Axillary bud and shoot tips of about 15 mm length obtained from sprouting rhizome buds were used. The excised explants were thoroughly washed with sterile distilled water and liquid soap followed by a brief immersion in a 70% ethanol. Subsequently, the explants were treated with 5% active chlorine concentration local bleach (Berekina), for 15 min under aseptic condition. To enhance the efficacy of the sterilant chemicals, two drops of Tween-20 (as a wetting agent) was added to each treatment. The explants were thoroughly rinsed (3 to 4 times) with sterile distilled water to remove remnant chemicals from explant surface.

Culture and their maintenance

Axillary buds and shoot tips of 10 mm approximate length were cultured on shoot multiplication media after few scale sheaths were removed.

Shoot multiplication

In each treatment, five jars with five explants per glass jam jars were used in each of the three replications laid in completely randomized design. MS basal medium with 20 g l^{-1} sucrose and 8 g l^{-1} agar at 5.8 pH were used. The cytokinins, N^6-benzyladenine (BA) and kinetin (N^6-furfuryladenine), were tested in combinations at four different levels (0, 1, 2 and 3 mg l^{-1}), from two explant sources, shoot tips or axillary buds as indicated in Table 1. The experiment was repeated twice to insure the reproducibility of the result.

Rooting

After six weeks of culture, explants with proliferating shoot clumps were cut into pieces and cultured in growth

regulator free media for a month to avoid carry over effect. Then after, 5 shoots were transplanted into glass Jam jar covered with modified plastic, and a hole in the middle sealed by sponge for gas exchange. The combinations of BA (0, 2, 4 and 6 mgl^{-1}) and α-naphthalene acetic acid (NAA) (0, 0.5, 1 and 1.5 mgl^{-1}) as indicated in Table 1 were used for root development. They were tested in an MS basal medium containing 30 g l^{-1} sucrose and 8 g l^{-1} agar laid in completely randomized design for rooting ability. All cultures were maintained at a growth room of 60- to 70% relative humidity and 25 ± 2°C with a 16 h photoperiod from cool white 40 W florescent bulbs supplying 2000 to 3000 lux light intensity.

Acclimatization

After the plantlets had developed roots and sufficiently elongated within four weeks, which was approximately 10 cm length, they were taken out from the culture vessel and treated with warm water for agar removal. Finally, 10 plantlets were planted in a plastic box of 2 L volume with a pre-sterilized potting mix of soil, sand and well decomposed coffee husk at a 1:1:1 ratio under 30% shade net condition for acclimatization according to Kambaska and Santilata **(**2009).

Measurements and data collection

Shoot multiplication data (number and length of shoot, number of leaves, fresh and dry weight of plantlets) were collected after six weeks of culture according to Kambaska and Santilata (2009). Fresh weight of plantlets were determined by removal from the medium, washed in distilled water and dried with filter paper before weighing. Dry weight was taken after drying the plantlets in an dry oven at a temperature of 65 to 70°C for 48 h at constant reading. Besides, data concerning root performance including mean number of roots and length were collected.

Data analysis

The data was analyzed According to Montgomery (2005) using SAS, statistical software package (Version 9.1) (SAS, 2001) and mean values were compared using REGWQ (Ryan-Einot-Gabriel-Welsch Multiple Range Test).

RESULTS AND DISCUSSION

With the use of local bleach, 70% contamination free cultures were obtained and used for subsequent experiments. Combinations of cytokinins resulted in considerable effects on various growth parameters associated with ginger shoot multiplication which is in line with the finding of Islam et al. (2004). Accordingly, very high significant difference ($p<0.0001$) were observed with regard to the number and length of shoots, number of leaves, as well as number of roots and fresh weight of plantlets. Moreover, there were highly significant differences ($p = 0.001$) in dry weight of plantlets with the aforementioned phytohormones combinations (Table 2).

Although, small quantity of cytokinin is synthesized by apical rhizome buds, its exogenous supply stimulates and promotes shoot proliferation in most of the plants (Ammirato, 1986). Similarly, it was observed that with increase in the concentration of BA from 0 to 3 mg l^{-1}, the number of shoot started to increase till it reached its optimum concentration. But, shoot parameters were also affected by mutual kinetin concentrations in association with BA. In line with this, the interaction effect between combinations of cytokinins with explant type showed a very high significant effect for shoot number ($P < 0.0001$). The variations observed in leaf number ($P = 0.008$) and shoot length ($P = 0.0024$) were also highly significant (Table 2). From the treatments, shoot tip explants supplied with 2 mg l^{-1} and 1 mg l^{-1} kinetin gave the highest average number of shoots (7.00 shoots per explant) followed by 3 mg l^{-1} BA and no kinetin (4.50 shoots) (Figures 1 and 3).

The interaction effect of cytokinins with explant type had also showed significant differences for both leaf number and shoot length. Plantlets developed from shoot tip explants on 3 mg l^{-1} BA alone gave majority of the leaves (5.33) followed by shoot tip with 2 and 1 mg l^{-1} kinetin) (5.17). With regard to shoot length, the combination of shoot tip explants with 2 and 1 mg l^{-1} kinetin) was the highest (5.85cm) followed by 1 mg l^{-1} BA and 3 mg l–1 kinetin (4.93 cm) (Figure 3).

Similarly, significant interaction effects were also recorded for fresh and dry weights of plantlets when culture media composition was evaluated together with explant type. Plantlets derived from 2 mg l^{-1} kinetin alone on axillary bud explants gave the highest fresh weights (0.685 g), followed by shoot tip explants cultured with 2 mg l^{-1} BA and 1 mg l^{-1} kinetin (0.58g). Regarding plantlet dry weight, the explants developed from shoot tip explant with 1 mg l^{-1} kinetin alone were the highest (0.0735g), followed by 2 mg l^{-1} BA with 2 mg l^{-1} kinetin from shoot tip explants (0.069g) (Figure 3).

In addition, the growth parameters evaluated in the current study, namely, shoot number, leaf number, shoot length and fresh weight were highly affected ($p < 0.0001$) by the type of explant used (Table 2). Accordingly, number of shoot and leaves, as well as shoot length and fresh weight of plantlets were considerably enhanced with the use of shoot tip explants for culture initiation than axillary buds (Table 2). Similar observations have been observed in in vitro culture of Curcuma by Shagufta et al. (2009). On the other hand, bud cultures were observed to take longer time to differentiate into shoots, elongate and attain an overall growth and development.

Kambaska and Santilata (2009) reported insignificant difference between two ginger cultivars, Suprava and Suruchi. Similarly, non-significant difference was observed between Yali and Boziab cultivars except for the numbers of leaves (Table 2). Therefore, from the two cultivars, Boziab had significantly ($P=0.0009$) more number of leaves than Yali. On the other hand, the similarities observed in the current study between the two cultivars for most growth parameters evaluated could

Table 2. ANOVA summary of the effects of hormone, explant and variety on varied growth parameters of ginger shoots *in vitro.*

Source of Variations	DF	Shoot number		Leaf number		Shoot length		Fresh weight		Dry weight	
		Mean square	Pr > F	Mean square	Pr > F	Mean square	Pr > F	Mean square	Pr > F	Mean square	Pr > F
Media	15	0.16561211	<0.0001	0.15069726	<0.0001	0.11706217	<0.0001	0.03573655	<0.0001	0.00524964	0.0010
Explant	1	1.66312995	<0.0001	2.01995996	<0.0001	0.33661988	<0.0001	0.09555827	<0.0001	0.00077224	0.3812
Variety	1	0.01687329	0.1943	0.47962057	0.0009	0.00470327	0.4503	0.00073577	0.1444	0.00201245	0.1659
Media*Explant	15	0.10159228	<0.0001	0.09444533	0.0080	0.02155194	0.0024	0.01252063	<0.0001	0.00342811	0.0089
Media*Variety	15	0.00497388	0.9327	0.05632951	0.1780	0.01333064	0.0810	0.00083147	0.0327	0.00136362	0.2454
Explant*Variety	1	0.00080291	0.7761	0.01181245	0.5930	0.00506983	0.4332	0.00064389	0.1702	0.00644526	0.0198
Media*Explant*Variety	15	0.00351455	0.9863	0.03227874	0.6895	0.01095535	0.1958	0.00251053	0.0810	0.00242821	0.0610
Coefficient of variation		7.85		5.39		6.38		6.01		7.31	

give wider usage of the protocol developed to micropropagate close relatives. This is contrary to the findings of Shagufta et al. (2009) on Curcuma varieties in vitro multiplication that showed a variation.

In this experiment, ginger explants performed better at lower kinetin concentration (1 mg l-1). As the concentration of kinetin increases in comparison with that of BA, shoot length was observed to increase, though they look thin and light green in color. Similarly, Balachandran et al. (1990) observed such poor growth and development of the plantlets and explained that it is due to high kinetin that inhibits the oxygen uptake of the cells. The author also reported that higher concentration of kinetin was not suitable for Z. officinale. However, cultures with higher levels of BA than kinetin showed a better shoot growth and development that are relatively robust and dark green in color. Due to these factors, explants supplied with 2 mg l^{-1} BA and 1 mg l^{-1} kinetin gave the highest well elongated shoots in the present study. In addition, this treatment also provided a comparable fresh and dry weight, which could be attributed to the higher numbers of leaves as well as increased shoot length.

In the present study, the best treatment combination for shoot multiplication cytokinin requirements of the two ginger cultivars was found to be 2 mg l^{-1} BA combined with 1 mg l^{-1} kinetin. On average, this hormonal combination was observed to give about 7.3 shoots per explant within six weeks of culture in Yali and 6.67 in Boziab, when the initial explants were derived from shoot tips. Best root development was observed from combinations of auxin and cytokinin with significant difference ($P<0.0001$) between treatments (Table 3).

There is an endogenous auxin accumulation to promote ginger rooting even though it is insufficient and provided a hardly grown rooting in the cultured shoots (Kambaska and Santilata, 2009). Similarly, Nirmal et al. (2005) found that the required quantity of auxins is high for better proliferation; thus, further increase in root number would be at the expense of shoot length. Accordingly, in this study, small number of root was observed in cultures on auxin free basal medium and/or at lower level of NAA treatments. This in turn would have a considerable impact on the survival potential of the plantlets at the later stage of growth after field transplanting.

Accordingly, medium supplemented with 1 mg l^{-1} NAA alone resulted in 8.75 roots with 2.95 cm length on average (Figures 2 and 3).

In the present study, rooted plantlets were transferred from culture bottles to plastic cups for their hardening, prior to their final transfer to the external environment. The observation showed good survival rate of in vitro grown ginger plantlets at 85% for ‘Boziab’ and 82% for ‘Yali’ (Figure 4).

Conclusion

In this study, appropriate explant type and suitable cytokinin combination for in vitro propagation of the recently released ginger cultivars (Yali and Boziab) was observed. Shoot tip explants on MS basal medium with 2 mg l^{-1} BA and 1 mg l^{-1} kinetin gave higher number of shoots, as well as better performing plantlets with respect to other

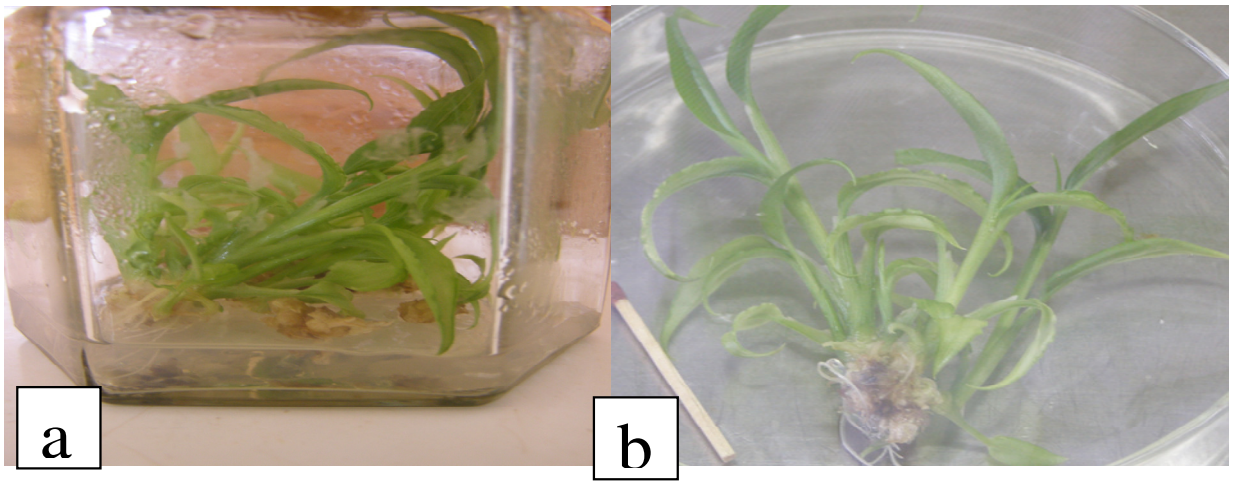

Figure 1. *In vitro* shoot multiplication of ginger from shoot tip explants after six weeks of culture: match stick length = 4.2 cm. (a) Shoot multiplication on 2 mg l^{-1} BA and 1 mg l^{-1} kinetin culture jar; (b) Observation of a single explant from 2 mg l^{-1} BA and 1 mg l^{-1} kinetin outside the culture jar.

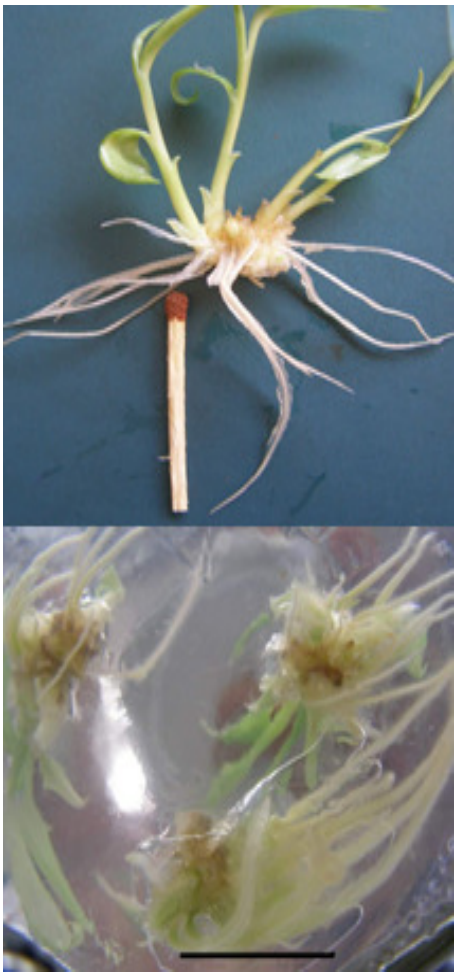

Figure 2. *In vitro* root development of ginger after four weeks of culture. Match stick length = 4.2 cm; bar length = 2 cm.

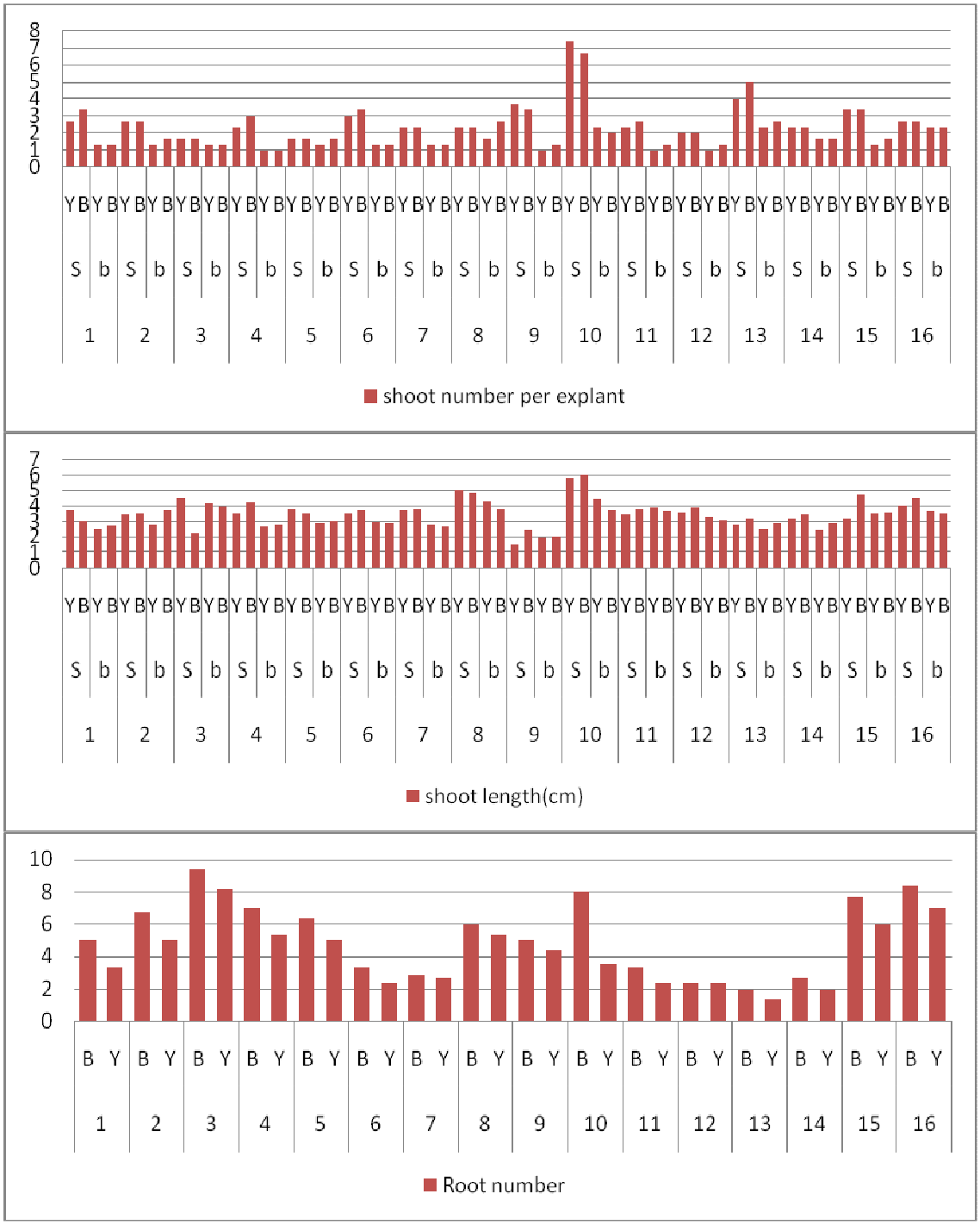

Figure 3. Growth regulator treatments and explant type effect on *in vitro* growth and development of two ginger variety plantlets; s = shoot explant, b = bud explant, Y = variety Yali, B = variety Boziab, 1 to 16 are media treatments as indicated in Table 1; n = 192.

growth and development parameters. This includes number of leaves and dry weight of plantlets regardless of varietal difference in comparison to axillary bud. Similarly, shoots cultured on MS medium with 1 mg l^{-1} NAA alone developed numerous longer roots. Plantlets produced using this propagation protocol was successfully acclimatized within four weeks of hardening period. This acclimatization procedure has been supported with

Table 3. ANOVA summary for *in vitro* ginger rooting.

Source of variation	DF	Root number		Root length	
		Mean square	Pr > F	Mean square	Pr > F
Media	15	27.0359375	<.0001	4.76244444	<.0001
Variety	1	36.8776042	<.0001	4.33500000	<.0001
Media*Variety	15	1.5498264	0.3036	0.77744444	0.0009
Coefficient of Variation		11.24		11.64	

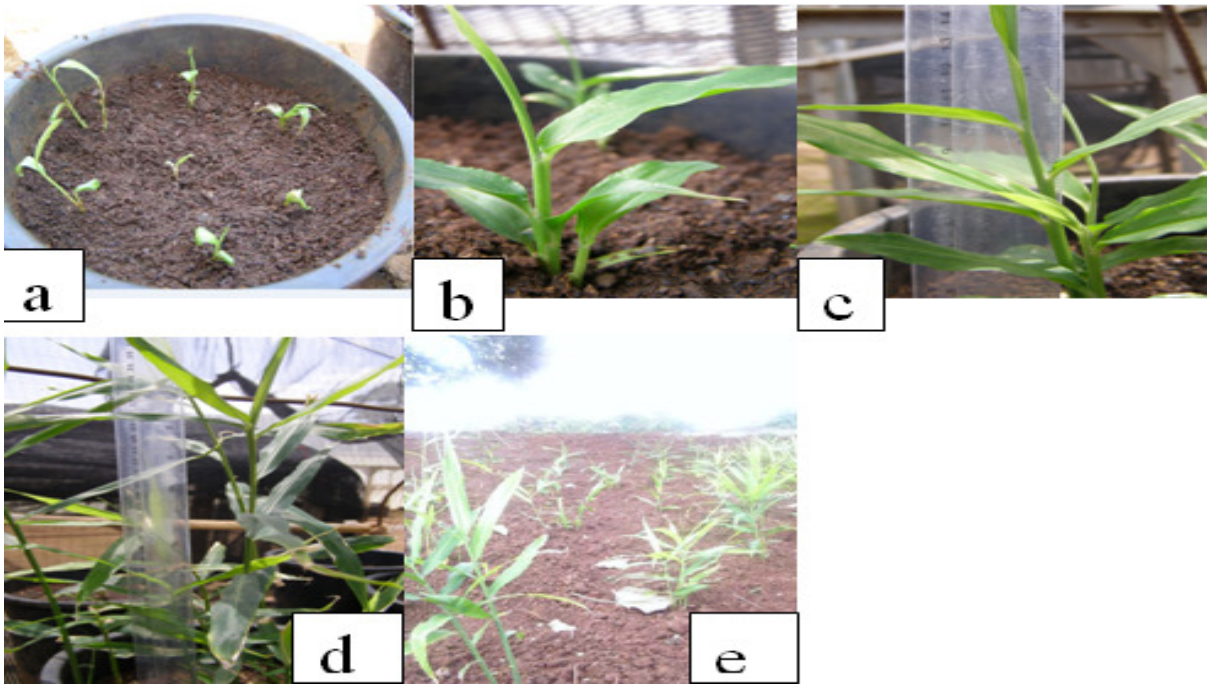

Figure 4. *Ex vitro* acclimatization of *in vitro* raised ginger plantlets; (a) Initial plantlets for acclimatization, (b) seedlings after two weeks, (c) seedlings after four weeks, (d) seedlings after six weeks, (e) seedlings survival under field condition.

the application of shade nets (at 30 and 70% shade level) and polythene under the green house condition. Subsequently, the seedlings have survived under field condition well.

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