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Full Length Research Paper

Environmentally safe *in vitro* regeneration protocol for *Curcuma*, *Kaempferia* and *Zingiber*

Dawood Ahmad[1,2]*, Noladhi Wicaksana[1,3], Takayoshi Shimazaki[1], Akira Kikuchi[1], Shakeel Ahmad Jatoi[4] and Kazuo N. Watanabe[1]

[1]Gene Research Center, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan.
[2]Institute of Biotechnology and Genetic Engineering (IBGE), NWFP Agricultural University Peshawar-Pakistan.
[3]Plant Breeding Laboratory, Faculty of Agriculture, Padjadjaran University, Indonesia.
[4]Plant Genetic Resources Program, National Agricultural Research Center, Islamabad- 45500, Pakistan.

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This study is a pioneer report on the development of an environmentally safe *in vitro* regeneration protocol for *Curcuma*, *Kaempferia* and *Zingiber*. The germplasm of the species was collected from Myanmar, a Southeast Asian country, rich in unexplored Zingiberaceae genetic resources. Rhizome buds were directly regenerated on the Murashige and Skoog medium containing a growth regulator, 6-benzyladenine and a commercial fungicide, Benlate (50% of Benomyl). The pre-treatment protocol did not contain $HgCl_2$, a toxic pollutant for *Curcuma amada*, *Curcuma longa*, *Zingiber barbatum* and *Kaempferia galanga*. Plantlets were regenerated from the buds without any intervention of the callus phase. The contamination free survival of the bud explants from *Curcuma*, *Zingiber* and *Kaempferia* was more than 75, 57 and 53%, respectively. Buds from immature rhizomes were difficult to regenerate on the media, as well as resulted in higher contamination percentages while the buds from mature rhizomes efficiently regenerated with very few contamination percentages. The contamination was in the range of 0 to 39% among the different accessions. This was also the first report of direct *in vitro* regeneration of plantlets from *Z. barbatum* bud explants. The protocol was cost-beneficial, time saving and effective for the conservation of Zingiberaceae genetic resources.

Key words: Conservation, regeneration, Zingiberaceae, tissue culture, *Curcuma*, *Zingiber*, Myanmar.

INTRODUCTION

The sustainable utilization and conservation of plant genetic resources is assisted and accelerated if collections are correctly evaluated, characterized and properly maintained (Hussain et al., 2008). Due to the fast occurring genetic erosion phenomenon, it is very important to conserve indigenous plant genetic resources representative of the prevailing genetic diversity especially from under-utilized agro-ecological regions. Field gene banks and *in vitro* gene banks are playing an important role in this perspective (Ravindran et al., 2005).

Zingiberaceae, known as ginger family, is one of the largest flowering families comprising 53 genera and more than 1200 species (Ahmad, 2010). Members of ginger family notably *Curcuma longa* and *Zingiber officinale* have been used for centuries in Chinese, Korean, Japanese and Indian cuisines and traditional Southeast Asian medicines. These species are important natural sources of spices, herbal medicines, dyes, perfumes and multipurpose aesthetic compounds (Chaveerach et al., 2008).

Myanmar, situated in Southeast Asia, is considered as one of the center of origin of Zingiberaceae plants (Ahmad et al., 2009). Myanmar is the habitat of 161 Zingiberaceae species belonging to 21 genera where *Curcuma* and *Zingiber* are the main species (Jatoi, 2008). The favorable climatic conditions and habitat diversity are the main reasons of plants genetic diversity in Myanmar (Ahmad et al., 2009). The Zingiberaceae genetic resources of Myanmar have not been well characterized and

*Corresponding author. E-mail: genesdoctor@yahoo.com. Tel: +92 91 9216553. Fax: +92 91 9216520.

Table 1. Description of the Zingiberaceae germplasm used in this study.

Accession number	Species name	Common name
ZO18-1	*C. amada*	Mango ginger
ZO23-1	*C. amada*	Mango ginger
ZO78	*C. amada*	Mango ginger
ZO112	*C. amada*	Mango ginger
ZO128	*C. amada*	Mango ginger
ZO129	*C. amada*	Mango ginger
ZO130	*C. longa*	Turmeric
ZO61	*K. galanga*	Lesser galangal
ZO113	*Z. barbatum*	Wild ginger
ZO31	*Z. officinale*	Ginger
ZO56	*Z. officinale*	Ginger
ZO84	*Z. officinale*	Ginger
ZO79	*Z. officinale*	Ginger

due to excessive uses and urbanization these valuable native plants need to be collected, characterized and properly conserved. In Myanmar, most of the Zingiberaceae species grow wild or as backyard plantation that demands conservation efforts (Jatoi et al., 2006, 2007; Ahmad et al., 2009).

Tissue culture is a common tool for conservation and rapid multiplication of asexually propagated species (Anish et al., 2008). Direct *in vitro* regeneration of explants without passing through callus phase, in order to keep integrity of the genome, offers a mean for conservation of vegetatively propagated plant species through tissue culture. Different *in vitro* culture protocols have been used for: *Z. officinale* (Bhagyalakshmi and Singh, 1988; Hosoki and Sagawa, 1977; Rout et al., 2001); *Kaempferia galanga* (Shirin et al., 2000); *C. longa* (Salvi et al., 2001, 2002); *Curcuma amada* (Prakash, et al., 2004; Barthakur and Bordoloi, 1992) and *Curcuma xanthorhiza* (Mukhri and Yamaguchi, 1986). Tissue culture for *in vitro* regeneration of the rhizome bud explants have not been previously reported for *Zingiber barbatum* species growing widely in Myanmar.

Most previously used protocols involved the use of $HgCl_2$ for disinfection of the explants. $HgCl_2$ is a serious environmental pollutant and potentially toxic to human and plants (Brandenberger and Maes, 1997; Ma and David, 2006). Ma and David, (2006) reported a disinfection protocol where $HgCl_2$ was not used for explants disinfection, but this protocol was used for a single species (*Z. officinale* only). This study reported a disinfection protocol exclu-ding $HgCl_2$ for explants pretreatment effective for *Curcuma, Kaempferia* and *Zingiber* the three very important genera of Zingiberaceae.

In Zingiberaceae, different plant organs have been used as explants; the most successful for direct regeneration are the rhizome buds (Ma and David, 2006; Prakash et al., 2004), but culturing efficiency of buds from “mature rhizomes” (rhizomes obtained after plants are harvested at the end of the season) and “immature rhizomes” (rhizomes obtained while plants are still growing in the field) have not been studied previously.

This study is a continuation of the previous efforts regarding characterization and conservation of Myanmar Zingiberaceae genetic resources (Jatoi et al., 2007; Ahmad et al., 2009). The specific objectives of this research work were; to extend the environmentally safe pretreatment protocol to other species of Zingiberaceae, to establish a single *in vitro* direct regeneration protocol effective for different species belonging to Zingiberaceae and to report on the culturing efficiency of buds from mature verses immature rhizomes. In addition, this study presented for the first time the *in vitro* plantlet production system for *Z. barbatum.*

MATERIALS AND METHODS

Plant material

Rhizomes of different Zingiberaceae species were collected from different sources in Myanmar. Detail descriptions of the germplasm are given in Table 1. These rhizomes were sown in pots in Tsukuba Japan; in a non replicated mode the growth period extended to 8 months (from end of April to end of December). The mother rhizomes produced new rhizomes referred here as “immature rhizomes” with new buds on them which were used as explants. At the end of the season plants were harvested in December and the rhizomes obtained referred here as “mature rhizomes”, were stored in the green house and were carefully checked for aeration and fungal contamination from time to time. In the month of March when the dormancy period came to an end, the mature rhizomes developed new buds which were also used as explants (Figure 1A).

Rhizomes pre-treatment

The buds from immature and mature rhizomes were used as explants depending upon their availability. The immature rhizomes buds were available in large number but only in the growing season when the plants were young. The mature rhizomes buds were

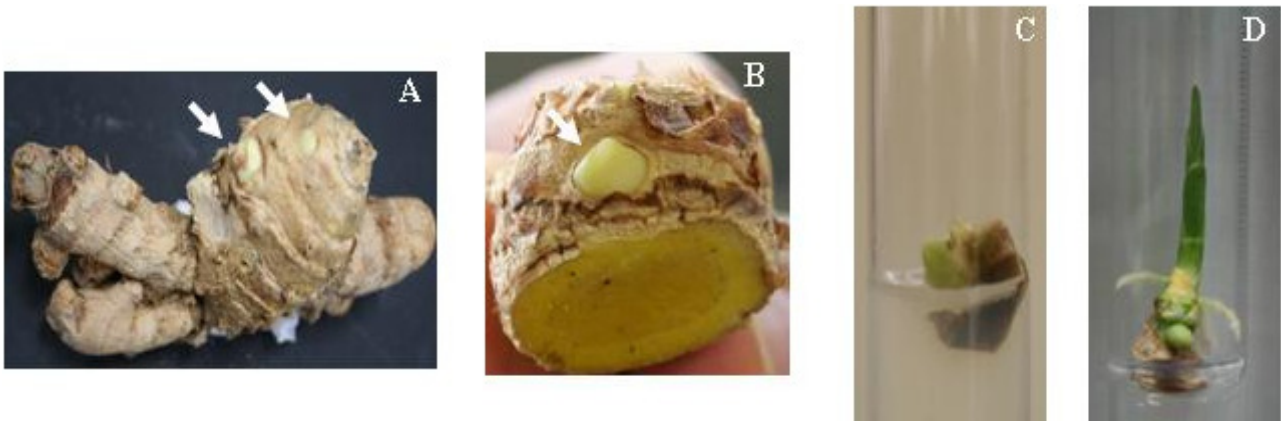

Figure 1. Different stages of buds explant culturing, (A) Mature *Curcuma* rhizome with newly developed buds at the end of the dormancy period; (B), Rhizomes piece possessing the bud explant to pass through disinfection before culturing; (C), a bud explant cultured on MS media; (D), a regenerated plantlet on MS media. The arrows indicate the buds explant on the rhizome.

available in small number but for longer time even when the rhizomes were not growing. The rhizomes were soaked in water, scarified and washed carefully. Each rhizome was sliced into small pieces (1 to 3 cm) with buds on them (Figure 1B). The rhizomes pieces were briefly treated with Tween 20 and rinsed with deionized distilled water for 5 min. Later on, the rhizomes were immersed in hot water (50°C) for 10 min. After the hot water treatment, rhizomes were shifted to clean bench and rinsed with autoclaved distilled water 4 to 5 times. Then, the rhizomes pieces were completely submerged in 10% common bleach (pH 12.6) for 30 min and rinsed once with autoclaved distilled water. The buds (5 to 10 mm) were cut apart from the rhizomes pieces. The buds were treated with 0.5% plant preservative mixture (PPM) for 10 min, rinsed in autoclaved distilled water, submerged in 70% ethanol and again rinsed with sterile distilled water.

Culturing conditions

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), containing 3% (w/v) sucrose and 0.8% (w/v) agar, was prepared with pH 5.6 ± 0.2. The media was microwaved till the agar was dissolved. When the temperature dropped down to 50°C the media was supplemented with a growth regulator, 6-benzyladenine and a commercial fungicide, Benlate (50% of benomyl, methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate) purchased from Sumitomo Chemical Garden Products Inc. Japan. Five ml media was poured into each test tube. The test tubes containing MS medium were autoclaved and later on stored at 4°C for subsequent use. The sterilized buds were cultured in the test tubes containing sterilized MS medium supplemented with benzyl adenine and benlate (Figure 1c). The test tubes were shifted to the growth room with room temperature maintained at 25 ± 1°C with 16 h light and 8 h dark period under 60 to 70% relative humidity in the culture room.

RESULTS AND DISCUSSION

Environmentally safe protocol

The use of metals free pre-treatment protocol is very important to avoid environmental contamination and insure health safety, especially for places where the dumping facilities of metals are not according to the internationally recommended standard. Heavy metals like mercury are known for immunotoxic and neurotoxin properties (Silva et al., 2005). Although, $HgCl_2$ is a very effective disinfectant in plant tissue culture explant pretreatment protocols, but it was shown in this study that its use is not imminent and can be avoided.

One of the novelties of this work was to establish a single *in vitro* direct regeneration protocol efficient for different species of Zingiberaceae simultaneously. Figure 2 represents the work flow of the protocol, from rhizomes harvesting to explant culturing, effective for *Curcuma*, *Kaempferia* and *Zingiber* species. Following this protocol bud explants from *Curcuma*, *Kaempferia* and *Zingiber* after sterilization, were successfully established on the MS media and were contamination free even after successive sub-culturing.

Minimizing explants contamination

The immature rhizomes were directly taken from the soil during the growing period of the plants while mature rhizomes were taken from the green house and therefore, needed to be surface sterilized to eliminate the micro-organisms present on the surface of the explants. The use of Tween 20 (20%) and sodium hypochlorite solution (5%) effectively controlled the exogenous contamination. Exogenous contamination can effectively be controlled by using surface sterilants and disinfectants (Bajaj, 1989).

Plants harbor endophytic fungi and bacteria which cause serious contamination of *in vitro* cultures (Sarasan et al., 2006). The explants were passed through a series of pre-treatment steps including hot water and plant preservative mixture (PPM) treatment which helped to minimize the endogenous contamination. Endogenous

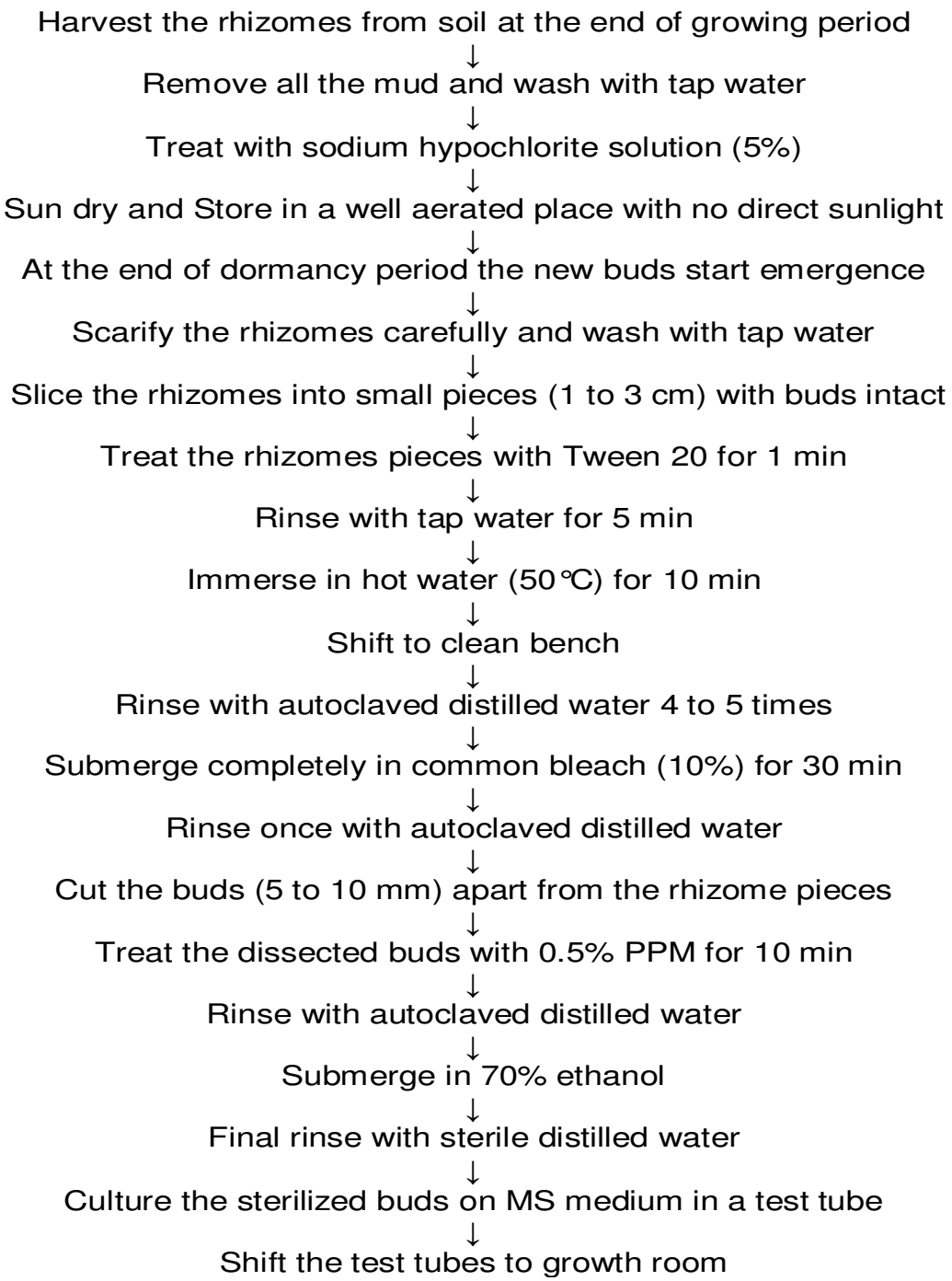

Figure 2. The work chart of the protocol effective for *Curcuma, Kaempferia* and *Zingiber* for the *in vitro* plantlet regeneration from rhizome harvest to *in vitro* culture.

contaminants can make their appearance in the culture even after a long period of time (Bajaj, 1989). To address this problem, Benlate (fungicide) and PPM was added to MS media.

The contamination was recorded for all accessions used in this study (Table 2). Bacterial contamination was a lesser problem compared to fungal contamination. The contamination percentage varied with a range of 0 to 39% for different accessions; while, the cultures of some accessions were totally free of contamination (Figure 3). Loc et al. (2005) showed 40% of zedoary explants cultures which were contaminated. In the case of *Zingiber,* only 10% cultures were contaminated (Ma and David, 2006). In this work, the overall explants contamination

Table 2. The contamination details of bud explants of the different accessions infected with bacteria and fungus.

Accession number	Explant cultured	Explant contaminated	Fungal contamination	Bacterial contamination	Contamination (%)
Curcuma					
ZO128	22	8	8	0	36.36
ZO78	26	1	1	0	3.85
ZO112	19	0	0	0	0.00
ZO129	14	0	0	0	0.00
ZO130	38	5	3	2	13.16
ZO23-1	12	1	0	1	8.33
ZO18-1	21	0	0	0	0.00
Total	152	15	12	3	11.76
Zingiber					
ZO113	16	4	4	0	25.00
ZO31	21	3	1	2	14.29
ZO56	32	3	13	4	14.29
ZO84	30	7	7	0	23.33
ZO79	33	13	5	2	39.39
Total	132	30	30	8	33.33
Kaempferia					
ZO61	15	1	1	0	6.67
Total	15	1	1	0	6.67

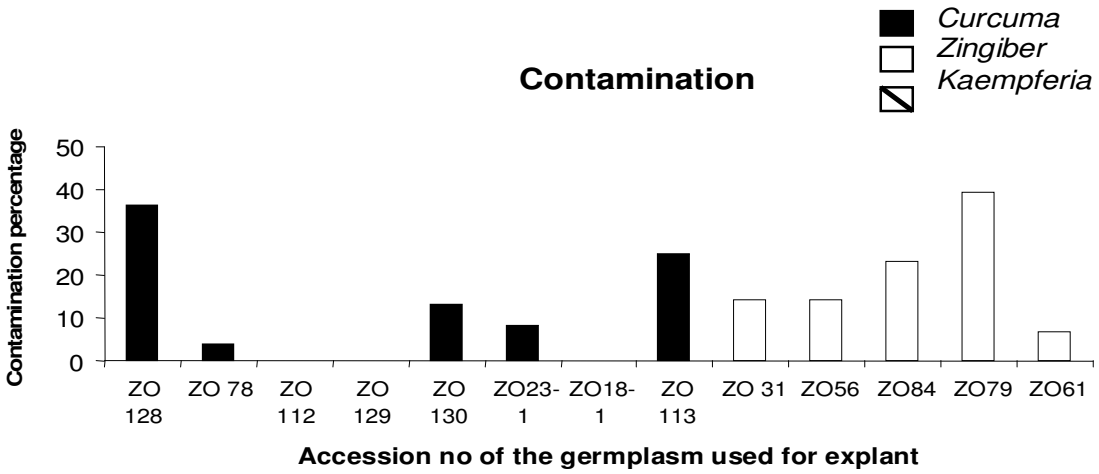

Figure 3. The comparison of the contamination percentages that occurred in the different accessions of *Curcuma*, *Zingiber* and *Kaempferia* after the explants were cultured on the MS media.

was less than 12% for *Curcuma,* less than 34% for *Zingiber* and less then 7% for *Kaempferia* (Table 2).

Bud-explants growth efficiency

The culturing efficiency of buds from mature rhizomes, which were stored for three months in the green house to pass through the dormancy period, versus immature rhizomes, which were obtained directly from the soil during the growth period of the plants, have not previously been studied in Zingiberaceae. When buds from the immature rhizomes were passed through explants pretreatment steps, these buds were unable to response to the MS media and most of them died (data not shown); the regeneration ranged from 5 to 20%. On

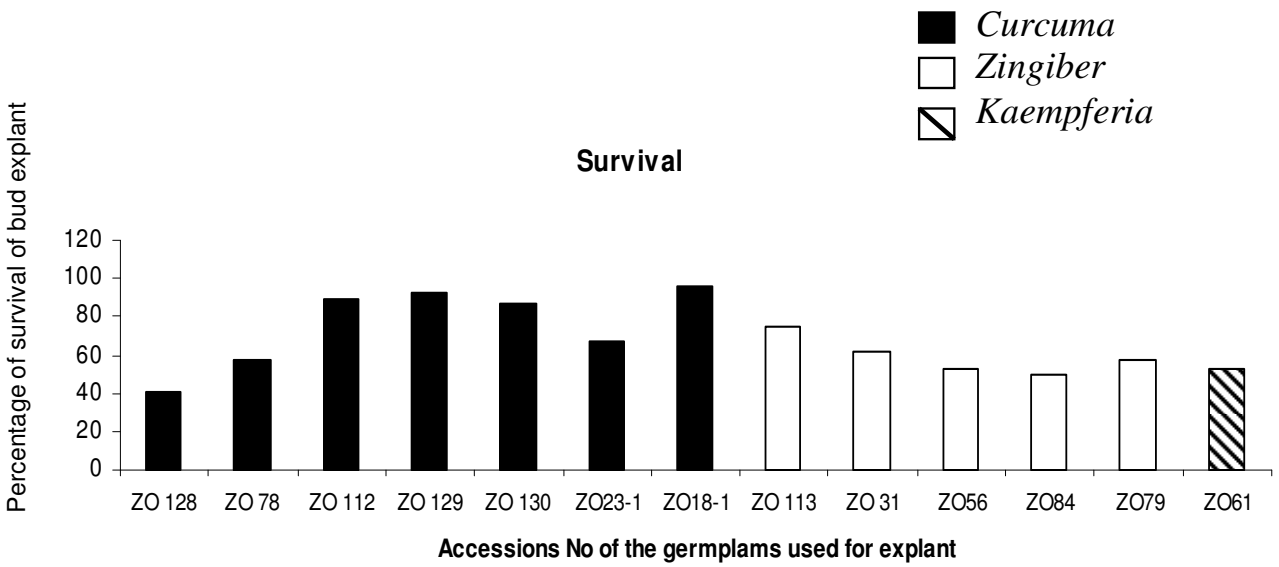

Figure 4. The comparison of explants survival percentages of different accessions of *Curcuma, Zingiber* and *Kaempferia* after the culturing on the MS media.

Table 3. The number of bud explants cultured from different accessions and their percentage of growth.

Accession number	Explant cultured	Explant that survived	Explant that died	Survival (%)
Curcuma				
ZO128	22	9	13	40.91
ZO78	26	15	11	57.69
ZO112	19	17	2	89.47
ZO129	14	13	1	92.86
ZO130	38	33	5	86.84
ZO23-1	12	8	4	66.67
ZO18-1	21	20	1	95.24
Total	152	115	37	75.66
Zingiber				
ZO113	16	12	4	75.00
ZO31	21	13	8	61.90
ZO56	32	17	15	53.13
ZO84	30	15	15	50.00
ZO79	33	19	14	57.58
Total	132	76	56	57.58
Kaempferia				
ZO61	15	8	7	53.33
Total	15	8	7	53.33

the other hand, when buds from the mature rhizomes were cultured in MS media, after passing through the same pretreatment protocol, they responded amazingly to the media and more than 40 to 95% buds started induction and produced contamination free plantlets (Figure 4). The data in this paper was based on the bud explants from the mature rhizomes. Figure 1 shows the type of buds selected from a mature *Curcuma* rhizome, the buds induction after culturing on MS media and the development of contamination free plantlet.

A variable number of bud explants both from *Curcuma* and *Zingiber* were cultured depending on the availability of the material (Table 3). The *Zingiber* buds explant started induction after two days of culturing, while it took 4 days for *Curcuma* explants to start induction on MS media. A total of 152 buds were planted for *Curcuma*

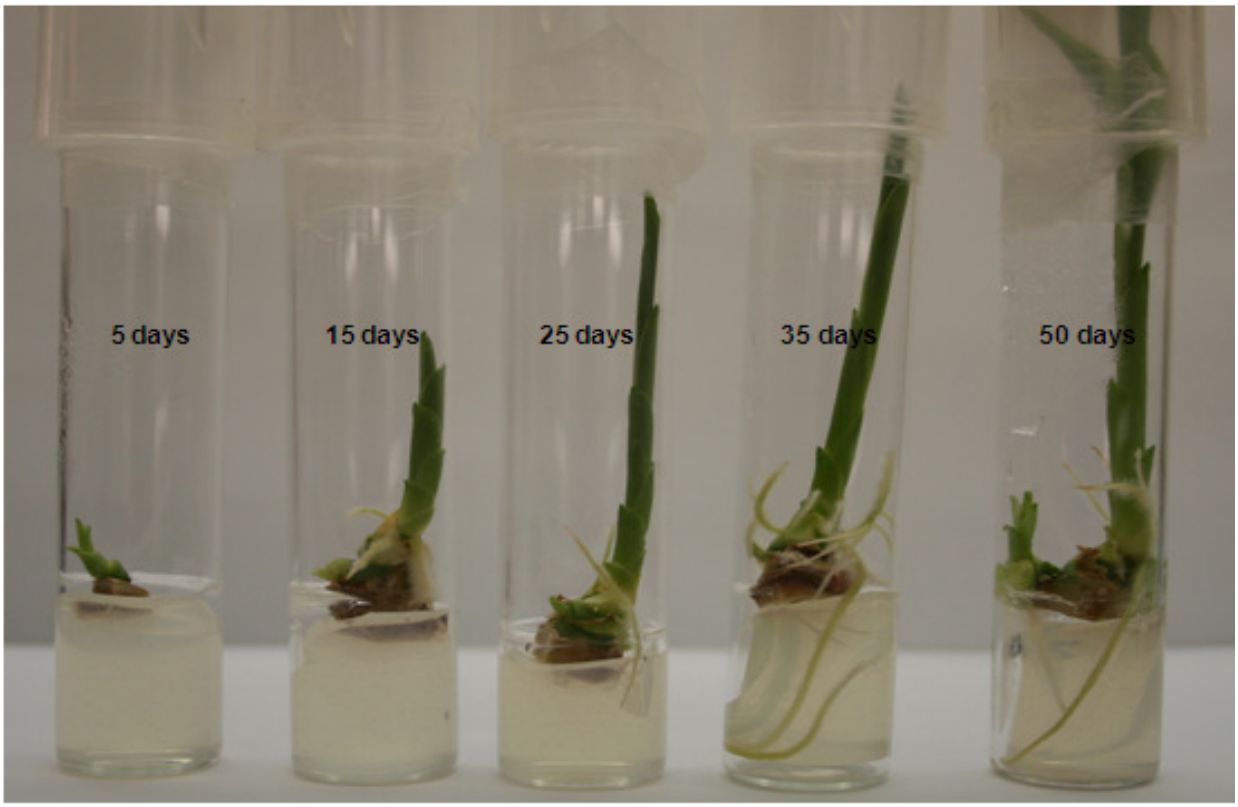

Figure 5. The regenerated *Curcuma* (ZO130) plantlets after buds were cultured on MS media in test tubes; the shooting started in the first week after culturing followed by rooting of the buds (the photographs were taken before changing the media each time).

where 115 grew contaminated free and produced plantlets, while the remaining 37 bud explant either did not grow or got contaminated (Table 3). In the case of *Zingiber,* 132 buds were planted where 76 buds grew contaminated free and made plantlets, while 56 buds did not grow (Table 3). In the case of *Kaempferia,* only 15 buds were planted where 8 buds grew contaminated free and made plantlets, while 7 buds did not grow (Table 3). In Zingiberaceae, different plant parts including buds, leaf sheets and inflorescence have been used as explants, where some studies reported rhizomes buds producing better results (Ma and David 2006; Prakash et al., 2004). The buds growth percentage varied from 40 to 95% in *Curcuma,* while this percentage decreased in *Zingiber* (53 to 75%). The *C. amada* accession ZO18-1 showed the highest survival percentage (Figure 4). Different stages of *in vitro* plants production including bud explants induction, shooting, rooting and growth at different days are shown in Figure 5.

Direct *in vitro* regeneration system for *Z. barbatum*

It is the first report on the *in vitro* plantlet production using *Z. barbatum* buds explants. The same protocol, used for other species in this study, was followed for *Z. barbatum in vitro* plantlet production (Figure 2). Unlike other species studied, Z. *barbatum* produced more plantlets (1 to 6) from a single bud where other species produced lesser plantlets (1 to 3) from a single bud in the test tube on MS media. Similarly, *Z. barbatum* explants survival rate was the highest (75%) among the other accessions of *Zingiber* (Table 3). The type of bud explants used, stages of shooting and rooting and the fully grown plantlet in the test tube on MS media are depicted for *Z. barbatum* in Figure 6.

Conservation through direct regeneration

As pointed out by Hussain et al. (2008), it is very important to select the representative diversity germplasm from fields and properly conserve them *in vitro*. Only a few Zingiberaceae species like turmeric and ginger are cultivated in some areas of Myanmar, while others like *Z. barbatum* and *C. amada* grow wild or are kept as backyard plantation. These plants are very essential not only to keep the integrity of the agro-ecosystem, but they are principal sources of traditional medicines. Due to the human activities including both urbanization and

Figure 6. Different stages of *Z. barbatum* buds explant culturing. (A), a mature rhizome with the arrows pointed to the buds emergence on the rhizome at the end of the dormancy period, These buds were passed through sterilization and then cultured on MS media; (B), emergence, shooting and rooting of the explants; (C), a 50 days old plantlet.

excessive use of the native plants without re-plantation, it is not only needed to collect these valuable genetic resources and characterize them but also their proper conservation is essential for their sustainable use. Being vegetatively propagated plants, plant tissue culture is a desirable method for their conservation. Direct regeneration is preferred for conservation through tissue culture in order to minimize soma clonal variation and genotype dependency which are likely to arise in the case of regeneration through callus, Tyagi et al., 2004). Using a single *in vitro* direct regeneration protocol for different species belonging to different genera of a family, can certainly reduce the cost and time needed for *in vitro* conservation. This protocol needs a further investigation to check the effect of different concentrations of different hormones in various combinations on the multiplication of the plantlets.

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