International Journal of Pharmaceutical Science and Research
ISSN: 2455-4685
Impact Factor: RJIF 5.28
www.pharmacyjournal.net
Volume 3; Issue 4; October 2018; Page No. 08-11

In vitro diseases cleaning, micro-propagation and field establishment of ginger (*Zingiber officinale* Rosc.) cultivars

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Abstract
Ginger (*Zingiber officinale* Rosc.) is an important tropical and sub-tropical herbaceous perennial plant widely cultivated spice in Ethiopia. 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 infected rhizome seeds. The morphologically good and healthy sprout buds were washed under running tap water supplemented with detergents followed by surface sterilization in 3%(w/v) $CuSo_4$ supplemented with three drops of Tween 20 for 15 min and rinsed with sterile water and the explants were treated by 70% (v/v) ethanol for 1min in laminar air flow hood, flashed again in sterilized water. Then, we were disinfected using 30% (v/v) sodium hypochlorite (5% active chlorine ingredient) for 15 min under laminar air flow hood. Therefore, *in vitro* disease cleaning and mass propagation of ginger were examined by biochemical and serological test via NCM-ELISA. The costumer ID of G_{04}, G_{06}, G_{10}, G_{15} and G_{17} were not showed (negative) results of ginger sample raised in *in vitro* Initiation, multiplication, rooting, Acclimatization and rhizome setting respectively. In addition to that, disease free ginger plantlets were mass propagated and commercialized to our customers.

Keywords: ginger, *Zingiber officinale*, *in vitro* cleaning, micro propagation, & NCM- ELISA

Introduction: Background

Ginger (*Zingiber officinale* Rosc.), a member of the family Zingeberaceae, is an important tropical and sub-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) [4]. According to the MOARD (2007) [9] report indicated that, ginger was cultivated in an area of 45,164 ha with production of 716,550 tons. According to the Ethiopian external trade statistics (2008) 22.6 million USD had been earned from ginger. The Authors, citing the report from Bureau of Agriculture and Rural Development (BoARD, 2008) reported that 2,896,372 Q of fresh ginger was produced from an area of 18,240 ha with average rhizome productivity of 160 Q/ha in the region (Q= quintal =100 Kg). For the past five consecutive years (2006-2011), Ethiopia stood 10th and 14th position in terms of area harvested and total production of ginger, respectively among the 36 countries engaged in ginger production globally (FAO, 2013) [5] and (Tadesse and Asfaw (2013) [10].

Although, there are more than 45 ginger cultivars in the country (MoARD, 2008) [8], According to Kavyashree *et al.*, (2009) [7] the production and productivity of ginger has been lost due to *Pseudomonas solanacearum* (bacterial wilt), *Fusarium oxysporum* (yellow leaf), *Pythium aphanidermatum* (soft rot), *Phyllosticta zingiberi* (leaf spot) and nematodes. In the current situation, Ethiopia has not benefited enough from ginger production due to remained partly traditional and bacterial Wilt being additional constraints. Moreover, micro propagation through conventional vegetative techniques is not recommended for infectious plant.

Micro propagation is an *in vitro* technique of growing aseptically plant cells, tissue or organs obtained from the mother plant in an artificially prepared nutrient medium. In addition to that, Micro-propagation by using tissue culture technique can be a proper alternative to produce disease free clones of ginger plant.

Justification

There is lack of access disease free planting materials. Conventional methods of propagating ginger via rhizome seed were not granted due to the incidence of bacteria wilt, inadequate agricultural management practice and dynamic nature of the pathogen. As a result, there is poor productivity and limitation for expanding ginger production making Ethiopia unable to benefit from its rich agro-ecological niche adopted to nationally release ginger cultivars. Moreover, the desirable traits of these nationally released ginger varieties production and distribution in a large-scale is difficult due to alarming of bacterial wilt. However, mass propagation of disease free ginger through tissue culture techniques is recommended and planted in clean and agro-ecologically adopted. In addition to that, true-to-type and disease free plantlets have been maintained *in vitro* using growth retardants. This study was assured that, *in vitro* mass propagation of disease free ginger (*Zingiber officinale*) cultivar through tissue culture techniques.

Research Methodology

This research project is planned to be conducted at Jimma, tissue culture laboratories, TNSRC on station and field evaluation in SNPPR, Oromiya, Amara, and new potential area of the country in the year of 2017 -2019.

Mother Plant collection and Establishments

National released elite cultivars of ginger were collected from Teppi National Spice Research Centre (TNSRC). The seed rhizomes is heat treated by 50ºC for 30 min in water bath and sowing in sterilized sand to maintain humidity in poly bags under lat house. Sprout bud 1 cm length samples for establishment of explants were obtained after four weeks of planted.

Surface sterilization of explants

The morphologically good and healthy sprout buds were washed under running tap water supplemented with detergents followed by surface sterilization in 0.3(w/v) mancozeb supplemented with three drops of Tween 20 and rinsed with sterile water, and then the explants were treated by 70% (v/v) ethanol for 1min in laminar air flow hood, flashed again in sterilized water. Then, they were disinfected using 30% (v/v) sodium hypochlorite (5% active chlorine ingredient) for 15 min under laminar air flow hood. Moreover, explants initiation cultured in hormone free media for two weeks and followed shoot multiplication in auxine and cytokinin as sole and/or combination fortified with full strength MS media for six weeks. Finally, disease free micro shoots were sub-cultured in ½ MS media for five weeks. Main while, the completed *in vitro* plantlets were acclimatized in the green house on the unsterilized 2:1:1 soil mixture of top soil, coffee husk and sand soil respectively for thirteen weeks.

Result and Dissection

Initiation

The sprout bud was cultured in Murashige and Skoog (MS) medium fortified with 30g/1sucrose, 7 g/1 agar and 1mg/1 N^6-benzyladenine (BA) in combination with 0.1mg/1naphthalene acetic acid (NAA). For cost effective initiation was germinated under hormone free media. The pH of medium had been adjusted to 5.75 prior to autoclaving at 121°C for 15 min. Therefore, Cultures were incubated in 25 ± 2°C followed by 16/8 h photoperiod conditions and the light intensity of 2000 lux provided by cool white fluorescent lamps.

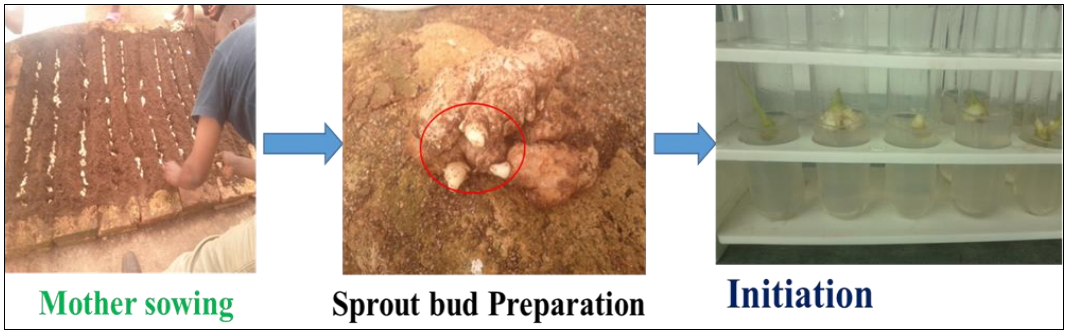


Fig 1: From left to right mother plant sowing, germinated bud and *in vitro* initiation cultures

Shoot Multiplication

The disease free sprout bud was cultured in Murashige and Skoog (MS) medium fortified with 30g/1sucrose, 7 g/1 agar and 2.5mg/1 BA in combination with 0.5mg/l kinetin had been obtained high multiplication factor and followed by 2mg/l BA in combination with 1mg/l kinetin in six weeks as you observed (table 1) and good morphology as you observe (Fig.2 B). However, the low multiplication factor in 3mg/l BA combination with 0.5mg/l NAA in eight weeks in (Table 1) was showed. Main while, these result was supported by (Biruk A. *et al* 2012) [1].

Table 1: The effect of Plant growth regulators for *in vitro* multiplication responses of ginger cultures

No.	MS media	PGR (Hormones)	Multiplication factor	Sub culture weeks
1	Full MS	2 mg/l BA + 1 mg/l Kn	6 Jars (5 explants/ Jar)	6 weeks
2	Full MS	2.5 mg/l BA + 0.5 mg/l Kn	8 Jars (5 explants/ Jar)	6 weeks
3	Full MS	3 mg/l BA sole	5 Jars (5 explants/ Jar)	7 weeks
4	Full MS	3 mg/l BA + 0.5 mg/l NAA	5 Jars (5 explants/ Jar)	8 weeks

*MS; macro, micro nutrient, vitamins, amino acids, 30 g/l sucrose, 7g/l agar and PH at 5.75

Multiplication and Rooting

The mass multiplication of disease free ginger was successively rooting was initiated at half strength MS media 15 g/l sucrose 7 g/l agar at four weeks. However, in shoot multiplication media rooting was initiated simultaneously. This protocol has been cost effective and time saving.

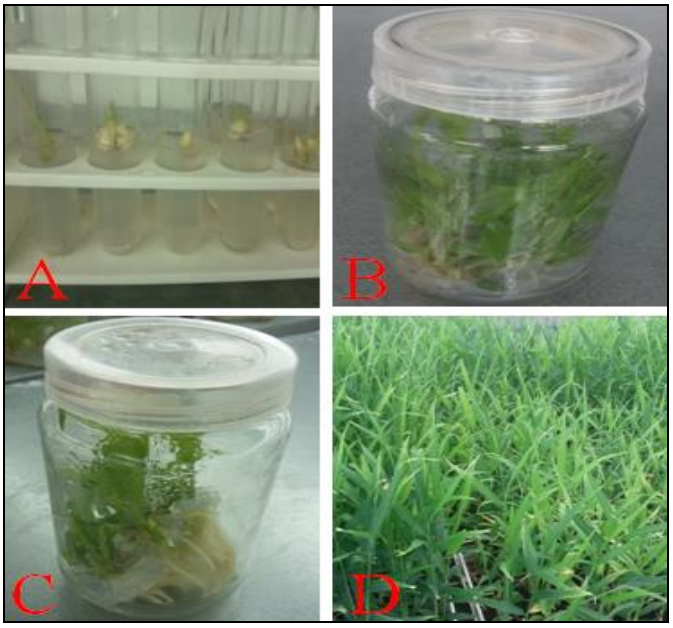


Fig 2: *In vitro* protocol optimization of ginger A) Intiation, B) multiplication, C) in vitro rooting and D) secondary acclimatiozaton in late house

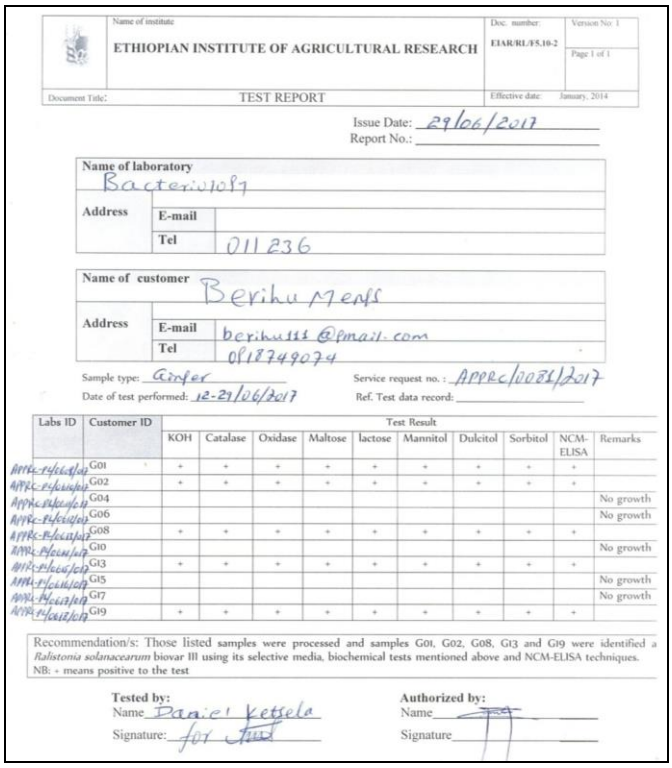
Acclimatization and Rhizome setting

For acclimatization, plantlets with well-developed root and leaf systems were washed with tap water to remove adhering media and agar attached on the roots of plantlets. Rooted micro-shoots were transplanted to plastic pots fulfilled with top soil, compost and sand (2:1:1) respectively. The plastic pots were placed in greenhouse where pot trays were covered with polythene to maintain humidity and evaporation for one week. Fortunately, the *in vitro* raised plantlets were acclimatized to outdoor conditions and kept in green house until plantation in the field.

Fig 3: A) primary acclimatization, B) Secomdary acclimatization in late house, C) on station planted seedling and D) rhizome setting after eight month harvested from field and pot seedling

Detection of *Ralstonia solanacearum* (bacterial wilt) of ginger via NCM-ELISA

Ginger bacterial wilt was diagnosed in Ambo Plant Protection Research Center (APPRC) through Biochemical and Serological test. Indeed, APPRC was accredited by Bacteriology department of International potato center (CIP) project



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Doc. number: EIAR/RL/F5.10-2 | Version No: 1 | Page 1 of 1

Document Title: TEST REPORT | Effective date: January, 2014

Issue Date: 29/06/2017
Report No.:

Name of laboratory: Bacteriology
Address
E-mail
Tel: 011 236

Name of customer: Berihu Menfs
Address
E-mail: berihu111@gmail.com
Tel: 0918749074

Sample type: Ginger
Date of test performed: 12-29/06/2017
Service request no. : APPRC/0021/2017
Ref. Test data record:

Labs ID	Customer ID	Test Result									
		KOH	Catalase	Oxidase	Maltose	lactose	Mannitol	Dulcitol	Sorbitol	NCM-ELISA	Remarks
	G01	+	+	+	+	+	+	+	+	+	
	G02	+	+	+	+	+	+	+	+	+	
	G04										No growth
	G06										No growth
	G08	+	+	+	+	+	+	+	+	+	
	G10										No growth
	G13	+	+	+	+	+	+	+	+	+	
	G15										No growth
	G17										No growth
	G19	+	+	+	+	+	+	+	+	+	

Recommendation/s: Those listed samples were processed and samples G01, G02, G08, G13 and G19 were identified a *Ralstonia solanacearum* biovar III using its selective media, biochemical tests mentioned above and NCM-ELISA techniques.
NB: + means positive to the test

Tested by: Name Daniel Ketsela Signature: for
Authorized by: Name Signature

*This paper was indicated for the laboratory result of ginger bacterial wilt. The costumer ID of G_{04}, G_{06}, G_{10}, G_{15} and G_{17} were not showed (negative) results of ginger sample raised in *in vitro* Initiation, multiplication, rooting, Acclimatization and rhizome setting respectively. However, the customer ID of G_{01}, G_{02}, G_{08}, G_{13} and G_{17} were showed that (positive) result of bacterial wilt of ginger sample were authorized.

Conclusion

Contamination by microbes had been a persistent problem for *in vitro* propagation of *Zingiber officinale.* Another serious constraint of micro-propagation of this plant is the presence of endophytic contaminations which are infected after wounding and cause subsequent symptoms and necrosis of explants. Therefore, *in vitro* mass multiplication, hardening off, field establishment of disease free ginger and finally Biochemical and serological test via NCM- ELISA were assured in Ambo National Plant Protection Research Center. However, contamination became more important for necrotic and rejuvenation of explants from aseptic bud was more difficult when compared to survival bud cultures. This protocol could also be an efficient procedure to eliminate contamination in monocot plants with higher potential for microbial contamination due to sprout apices being close to the ground.

Acknowledgements

The authors would like to acknowledge Jimma Biotechnology laboratory staff members for their advice and technical

support on reducing bacteria wilt of ginger by using aseptic techniques of *in vitro* culture. Highest gratitude is also extended to Ethiopia Institute of Agricultural Research (EIAR) for financial support and Ambo National Plant Protection Research Center (ANPPRC) for Laboratory result of Biochemical and Serological test of bacterial wilt of the ginger samples.

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