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In vitro Regeneration of Disease Free Enset (Ensete ventricosum (Welw) Cheesman) Planting Materials from Bacterial Wilt Diseased Plants Using Shoot Tip Culture

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Abstract
Enset is an important food crop produced in Ethiopia with great role in food security. The demand of the crop is increasing throughout the country. However, the production as the whole is decreasing due to devastation by enset bacterial wilt. *The studies were conducted to develop the procedure for obtaining* multiple disease free plantlets from infected enset plants. Shoot tip explants from infected suckers of three clones Arkiya, Digomerza and Mazia were cultured on MS media supplemented with different combinations of benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) each with concentration of 0, 1.5, 3, 4.5 and 6 mg/L with 0, 0.5, 1, 1.5 and 2 mg/L, respectively. The effect of growth regulators on shoot growth parameters was examined. The minimum days (11.66) for multiple shoot induction were recorded for Mazia on media with 4.5 mg/L BAP and 1.5 m/L NAA. The maximum number of shoots (23.0) was also obtained for Mazia on the same hormone combination as for days of induction. Whereas, the maximum shoot length (8.1 cm) was recorded for Digomerza on media with 3 and 1 mg/L NAA. Similarly, for root induction and growth MS media with different concentrations of indulebutyric acid (IBA; 0, 0.5, 1, 1.5 and 2 mg/L) were evaluated. The minimum days (10.5) to root induction was observed for Mazia on media with 1.5 mg/L IBA and the maximum root number (3.8) was recorded at 2 mg/L IBA. In the efficiency of shoot tip culture for Xanthomonas pathogen elimination, sample suspension was prepared from shoots regenerated from diseased suckers and transferred on semi-selective yeast peptone sucrose agar (YPSA) medium. The result of colony observation indicated that many microbes are living in enset saprophytically as mixed colony growth was observed within 24 h after sample culturing. Pathogenesity test on clean suckers of susceptible clone showed that the colonies grown were due to endophytic microbes since none of the colonies were capable to develop disease symptoms as sample of the pathogen strain.
Keywords: Ensete ventricosum, benzylaminopurine (BAP), indulebutyric acid (IBA), naphthaleneacetic acid (NAA), shoot tip, Xanthomonas, yeast peptone sucrose agar (YPSA).

INTRODUCTION

Enset (*Ensete ventricosum* (Welw.) Cheesman*)* is a diploid (2n=18) herbaceous perennial edible species of the separate genus of the banana family, thus named 'false banana'(**Chessman ,1947)..** Enset is the vernacular name used in the Amharic language in Ethiopia for *Ensete ventiricosm* whose fruit is not edible and one of the indigenous root crops widely cultivated for its food and fiber values **(Taye, 1996).** Variation within the species to altitude, soil and climate has allowed widespread cultivation in the mid to highlands of Southern and South-Western part of the country. It is estimated that a quarter or more than 20 million of Ethiopia's population depends on enset as staple and co-staple food source, for fiber, animal forage, construction materials and medicines (Zerihun et al., 2014). and the area of enset production in Ethiopia is estimated to be over 321,362.43 **hectares (CSA, 2013).** The average yield of kocho (non-dehydrated fermented product from the mixture of decorticated pseudo stem, pulverized trunk and corm) of superior food and fiber yielding cultivars such as Digomerza gave yield over 43 ton per hectare bases per year (Atnafua et al., 2008). Fresh enset parts are used as fodder for domestic animals during dry season and some enset clones are reported to have medicinal value to human beings and domestic animals (Temesgen et al., 2014). Enset is also well known to conserve soil and enrich plant nutrients through its dropped foliage, dried leaf sheath and petioles of enset are used for wrapping materials and other utensils (**Endale and Mulugeta, 1994).**

Due to limited research attention given to enset crop, its production system is still traditional and tiresome. Different management practices starting from propagation to harvesting and processing demand high labor. Various diseases and insect pests of enset also have been reported. Some of these are: leaf damaging fungal diseases, corm rot, sheath rot and dead heartleaf rot of enset with unknown causal agents and root knot, root lesion and black leaf streak nematode diseases. There are also viral diseases of enset known as mosaic and chlorotic leaf streak diseases. Insects damaging enset leaves such as Jassid fly, spider mites, mealy bugs and wild animals such as porcupine, mol rat and wild pigs have been reported (Brandt et al., 1997). However, based on the distribution and the damage incurred on enset production, enset bacterial wilt disease caused by *Xanthomonas campestris* pv. *musacearum* is known to be the most threatening and important problem to enset production system.

The disease was first reported and described by Dangnachew and Bradbury (1968) that attributed it to *Xanthomonas musacearum* (Eshetu, 1981). It was widely distributed in many enset growing regions of the country and affects the crop at all developmental stages and the results obtained from recent bacterial wilt disease assessment made in some enset fields in Southern Nations, Nationalities, and Peoples Region (SNNPR) showed losses of up to 100% under severe damage **(Anonymous (Awassa Agricultural Research center), 2008).** Natural epidemics of the disease were also observed in banana fields at different enset growing areas. Even though the disease is widely distributed and important, there are limited research efforts directed to manage the disease using cultural practices and enset clonal screening against bacterial wilt which were hindered due to knowledge gap from farmers and tedious destructive work to farmers to remove all infected plants from field in which sometimes the whole farm is infected. There is no clone still that can resist the pathogen after artificial inoculation by different researchers with conflicting reports for the same clones interaction to the pathogen strains which have variations on pathogenicity as well as biochemical reactions (Kidist, 2003; Gizachew, 2008).

Attempts by recombinant DNA technology to develop genetically improved enset clones with resistant gene are absent due to lack of detailed genetic information (Morpurgo et al., 1993). Different researchers have tried to develop protocols for micro propagation of clean planting materials to distribute for end users, which indicated that there is a possibility to produce large amount of *in vitro* plantlets with further work needed in the basic physiology of the crop to verify efficient micro propagation protocol (Mulugeta and Tesfaye, 2010). Recently, shoot tip/meristem culture has the potential to eliminate viral, fungal and bacterial diseases from plants. In the closely related crop, banana, tissue culture effectively eliminates weevils and nematodes, bacterial diseases such as the Banana *Xanthomonas* wilt (BXW) and fungal diseases like *Fusarium* wilt and Sigatoka (Kabir et al., 2008). Tissue culture has been also shown to bring down the cost of controlling foliar diseases by half. However, there is no information either for the possibility of generating disease free suckers from infected enset plant explants.

Therefore, the objective of this study was to optimize *in vitro* protocol for enset bacterial wilt free planting materials production and finally test for pathogen absence using different diagnostic techniques.

MATERIALS AND METHODS

Experimental materials

Three month old suckers of three clones of enset were collected from Areka station and maintained under greenhouse conditions until they resume growth. After one month these suckers were inoculated by Hagereselam strain of *Xanthomonas* and later used as explants source for culture initiation.

Explants surface sterilization and culture initiation

Explants with 2 cm were collected from sucker corms (Figure 1) and thoroughly washed with detergent and sterile distilled water (SDW). Then, the explants were treated with 70% of ethanol for 5 min followed by double sterilization with 40% of gion berekina (2% NaOCl) for 10 and 20 min accompanied by 4x rinse with SDW between each treatment. Finally, the size of the explants was trimmed to appropriate size (1 cm) and inoculated to initiation medium as per treatments.

Shoot induction and multiplication

Surface sterilized explants were cultured in jars containing 40 ml of shoot initiation MS medium (Murashige and Skoog, 1962) with 3% of sucrose, 0.6% of agar and 0.3% of activated charcoal (AC). MS media supplemented with different combinations of benzylaminopurine (BAP) and naphthaleneacetic acid (NAA) as per treatmet. All treatments were maintained in dark condition for fifteen days at initiation stage to enhance explants establishment by reducing blackening of explants due to phenolic exudation and preventing formation of other growth inhibitors in the media as recommended by Pierik and Steegmans (1975). Sub-culturing of initiated cultures for multiplication was done after one month in multiplication medium supplemented with different combinations of growth regulators as in shoot initiation. Multiplication stages were maintained in controlled growth room set at average of 27 ± 2°C temperature, 60 to 70% of relative humidity (RH) and 40 µmol/m^2s of light intensity under 14/10 h light and dark period. While sub-culturing the middle growing shoot tip was carefully excised together with the lower corm base part and transferred to multiplication medium supplemented with different concentration and combination of growth regulators. After one month of incubation in growth room, shoots with length of 1.5 cm and more were carefully separated from the explants and transferred to regeneration media. However, explants with smaller shoot buds that cannot grow independently but potentially grow to shoots were transferred to the same multiplication media to get more shoots in the second sub-culture.

Root induction

Elongated individual shoots with leaves were carefully separated from explants and cultured in 250 ml jars containing 40 ml of rooting MS media supplemented with 0, 0.5, 1, 1.5 and 2 mg/L of indulebutyric acid (IBA). Similar to multiplication stages, cultures were maintained in controlled growth room.

Diagnostic techniques to test pathogen elimination

Bacterial sample preparation

Sample suspension was prepared from well regenerated *in vitro* plantlets leaf and leaf sheath section at which *Xanthomonas* was believed highly available. The sample plantlets were taken from growth medium by forceps and the lower corm part with roots was cut down and the upper part was placed in sterile distilled water. The sample shoots were then crashed carefully in order to make bacterial cell suspension if available within plant tissue in 1 ml of distilled water. The suspension sample was then serially diluted five times by 1:9 ratio of sample and distilled water, respectively.

Semi-selective medium preparation

Semi-selective culture media yeast peptone sucrose agar (YPSA) for the pathogen was prepared from 5 g/L of yeast extract, 10 g of peptone, 10 g/L of sucrose and 15 g/L of bacteriological type agar for 1 L media as in the case it was prepared for initial inoculums preparation by dissolving the mixture of media components together on hot plate with magnetic stirrer. The media solution was autoclaved in pressure cooker for 25 min under 1.2 $kgms^2/m^2$ and 121°C. Finally, 20 ml of the hot media was dispensed to sterile Petri dishes and stored in refrigerator at 15°C for two days.

Plant extract sample inoculation to semi-selective media and colony observation

All petridishes were labeled according to sample code and replicated twice. The suspension sample was then stricked on the media by loop. The loop was sterilized in the intervals of samples stricking. For negative and positive control two petridishes for SDW and two petridishes for bacterial sample, respectively, were used. Then petridishes with samples were sealed by plaster and taken to incubator set at temperature of 27°C. After incubating overnight, cultures were checked for any colony growth on the media including the controls.

Pathogenesity test

Pure colonies were taken by sterile loop from mixed colonies grown on semi-selective media and sample suspension was prepared by serial dilution technique to 10^8 cfu/ml. The suspension was then inoculated to healthy growing suckers of Arkiya which has shown high susceptibility to check that the grown colonies are of *Xanthomonas* pathogen or not. Artificially, inoculated plants were observed for disease symptom development in seven days interval up to 35 days.

Acclimatization

After four weeks of transferring shoots to rooting medium, well regenerated plantlets with shoots, roots and leaves were gently removed from the culture jars and the roots were washed in tap water to remove traces of agar. The plantlets then were transplanted into dark plastic pots filled with sterile soil mix of 1:1 red ash and sandy loam soil under green house. The pots in which plants were planted for acclimatization were finally covered with white polyethylene bags and red cheesecloth to maintain high humidity for fifteen days.

Data recording

Data for explants response to treatments such as number of explants contaminated on initiation mean number of days to shoot and root induction, mean number of shoots, number of leaves and roots per plant, shoot and root length and fresh and dry weight of shoot and root were recorded.

Data analysis

The experiment was designed in CRD and laid in factorial arrangement with three replications for all treatments. Data recorded for all responses were subjected to analysis of variance (ANOVA) and significant differences among treatments were determined by Fisher's Least Significance Difference test (LSD) using SAS software (JMP version 9.2) for the differentiation of the effect of treatment, genotype and treatment-genotype interaction.

RESULTS AND DISCUSSION

Surface sterilization of explants

Initial surface sterilization experiment was effective when 2 cm shoot tip explants were treated with 70% ethanol for 5 min followed by double sterilization with 40% of gion berekina (2% chlorox) first for 10 and then 20 min. This treatment resulted in 95% contamination free explants and very less (2%) dead explants for all the clones until 10 days of inoculation in MS media. Later when explants started to develop single shoot let bacterial colonies started to develop under the explants in both pathogen inoculated and healthy mother plant sourced explants which led to loss of many cultures. Combination of two antibiotics, namely ampicilin and gentamicine with the concentration of 30 mg and 10 mg, respectively, per 100 ml of distilled water was used for decontamination by immersing surface sterilized explants for 2 h before transferring to initiation media. Similarly, Bermita (2004) reported that microbial contamination, in particular by apparently endophytic microbes that are resistant to antimicrobial agents was encountered during micro propagation work of enset.

Effect of BAP and NAA concentration on shoot induction and growth of enset clone explants Days to single shoot initiation

Remarkable variation was observed for days required for shoot initiation among the three clones and 5 levels of hormones (BAP with NAA) combinations. The minimum days required for shoot initiation (5.25 days) was noted in Arkiya at 6 mg/L BAP and 2 mg/L NAA in combination, Digomerza (5.50 days) on the same hormone combination as in Arkiya and for Mazia (5.50 days) on media with 4.5 and 1.5 mg/L of BAP and NAA (Table 1). Whereas, the maximum days required for single shoot initiation for Mazia (9.00) and (8.67) were recorded on hormone free and media supplemented with 1.5 mg/L BAP and 0.5 NAA, respectively, followed by Digomerza (8.33) and Arkiya (8.25) on hormone free MS media (Table 1). Mulugeta and Stedany (2004) reported that, single shoot initiation observed after two weeks when enset shoot tip explants cultured on MS media gelled with 11 g/L of agar and supplemented with 2.5 mg/L BAP alone. The most probable reasons for this early initiation in the present experiment were the low amount of solidifying agent used that can reduce nutrient uptake by explants if in large amount since the media become very compact and the combined the effect of BAP and NAA.

Days to multiple shoot induction

Considerable variation was observed in days to multiple shoot induction among clones cultured on different concentration of growth hormones. The minimum days to multiple shoot induction was recorded for Mazia clone (11.66 days) cultured on media with 4.5 mg/L BAP and 1.5 mg/L NAA followed by 12.67 days for the same clone explants cultured on 6 and 2 mg/L supplemented MS media. On the other hand, the maximum days to multiple shoot induction was noted more for Arkiya (25.33) and Digomerza (23.33) followed by 23 days for Mazia explants cultured on hormone free MS media (Table 1). So far there is no report on enset for mean days to multiple shoot induction *in vitro* after sub-culturing initiated explants in multiplication media. Hirimburegama and Gamage (1996) conducted experiment to develop micro propagation protocol for ten cultivars of close related species banana. They have reported that different cultivars of banana shoot tips cultured for shoot proliferation has induced multiple shoots at different times after inoculation to MS medium supplemented with BAP and IAA at different level which agreed to the present finding.

Number of micro-shoots per explants

Mazia was the best performing clone in terms of shoot numbers (23.00) proliferated per explants on media with 4.5 mg/L BAP and 1.5 mg/L NAA followed by Digomerza (20.33) on media containing 6 mg/L BAP and 2 mg/L NAA. On the other hand, Arkiya has responded poorly for number of micro-shoots to all combinations of BAP and NAA as compared to the other two clones Whereas, explants cultured on hormone free media has produced the smallest number of shoots for the three clone explants (Table 1 and figure 2). Earlier, a procedure for micro propagation of enset has been reported by Negash et al. (2000), but only 2 to 3 shoots were produced from a single explant. Similarily, Mulugeta (2004) obtained 3.7 shoots and 3.5 shoot buds per single shoot after splitting the explants in two and culturing them separately. In spite of the limited response of the crop *in vitro,* Bermita and Welander (2004) reported about 75 shoot buds that can potentially grow to shoots per explants in one sub-culture through meristem wounding of initiated explants. However, the regeneration capability of the protocol she had developed was reported later has draw back (Mulugeta and Tesfaye, 2010). The result of the present experiment showed that the maximum mean number of shoots obtained was 23 for Mazia clone on media with 4.5 mg/L BAP and 1.5 mg/L NAA in one sub-culture that can be transferred to regeneration media directly and more than 30 buds that can potentially grow to shoots in second sub-culturing to the same multiplication medium.

Shoot length per plantlet

Remarkable variation was observed for length of shoots among clones and in different hormone combination. The maximum shoot length was recorded for Digomerza (8.06 cm) on media with hormone combination of 3 mg/L BAP and 1 mg/L NAA followed by 7.46 and 7.36 cm for Mazia and Digomerza, respectively, on media supplemented with 4.5 mg/L BAP and 1.5 mg/L NAA (Table 1 and Figure 3A to D). Whereas, the lowest shoot length (1.5 to 2.73 cm) was recorded for all the three clone shoots developed on media with 6 mg/L BAP and 2 mg/L NAA followed by shoots from hormone free MS media (Table 1 and Figure 3). In the current study, the appropriate concentration of BAP and NAA that resulted in maximum shoot length is 3 and 1 mg/L, respectively, for all the three clones. Because further increase up to 4.5 mg/LBAP and 1.5 mg/L of NAA did have any significant effect and has reduced shoot length for all the three clone explants, indicating the upper limit in concentration (Table 1).

Leaf number per shoots

Among the three clones tested the highest mean number of leaves (2.80) per plantlets was recorded for Mazia clone followed by 2.66 for Digomerza clone. Whereas, the lowest number of leaves (2.06) were recorded for Arkiya (Table 2). The maximum leaf number (4.11) was recorded for explants cultured on MS media supplemented with 4.5 and 1.5 mg/L BAP and NAA hormone combination followed by 3.77 leaves per shoot on 3 and 1 mg/L NAA supplemented media (Table 2). Whereas, the lowest mean leaf number was recorded for explants cultured on hormone free MS media (1.00) that looks like leaf primordial rather than normal leaf with

stunted growth (Table 2 and Figure 3). In comparative study for effect of explants source on plant regeneration, Mulugeta and Stedany (2004) have reported that three to four leaf per shoot for *in vitro* source explants and two to three leaves for shoots regenerated from greenhouse source explants. In the present study, the experimental material was from green house grown suckers that were artificially inoculated by bacterial wilt pathogen, but the result was in agreement with that of number of leaves recorded for shoots grown from *in vitro* source explants.

Effect of IBA concentration on root induction and growth in explants of the three enset clones

Number of days to root induction

The minimum number of days to root induction was recorded for Mazia (10.50 days) and Digomerza (11.83 days) for shoots cultured on media supplemented with 1.5 mg/L of IBA followed by Arkiya (12.66 days) on media with 1 mg/L IBA and Digomerza (12.83 days) on media containing 2 mg/L of IBA. Whereas, the maximum mean days to root induction was recorded on control hormone free media for all clone roots (Table 3). Negash et al. (2000) reported that root formation occurred in less than two weeks after transfer to root induction medium supplemented with 5 µM IBA, 1 µM IAA and 1 µM BAP in combination for all three enset clones on which they have conducted their experiment which is in agreement with the present finding.

Number of roots induced per shoots

The maximum root number (3.77) was recorded on MS media supplemented with 2 mg/L of IBA followed by 3.11 on media with 1.5 mg/L of IBA (Table 5). Whereas, the lowest mean number (1.44) of roots per shoot was recorded for roots induced on hormone free MS media. Experiment on clonal propagation of wild type *Enset superbum* (Roxb.) Cheesman conducted by Mathew and Philipe (1996) recorded 6 to 7 adventitious roots per shoots transferred to half strength MS media supplemented with 3 mg/L IBA and 0.5 mg/L BAP after one month of incubation in growth room. The result of the present experiment revealed smaller number of roots per shoot which might be due species difference and high concentration of IBA they have used.

Length of roots induced per shoots

The maximum mean root length was obtained from shoots cultured on media with the lowest concentration of IBA (0.5 mg/L) for Digomerza (5.96 cm) followed by Mazia (5.03 cm) cultured on the same IBA concentration and 4.96, 4.90 and 4.80 cm for Digomerza shoots transferred to media with 1, 1.5 and 2 mg/L of IBA, respectively, with no significance difference (Table 4 and Figure 4A to D). On the contrary, the lowest root length (2.50 cm) was recorded for Mazia on hormone free media followed by Arkiya (3.20 cm) on the hormone free media after four weeks of culturing (Table 3). Digomerza has responded better for root length as compared to the two clones whereas, Arkiya responded poorly. The optimum level of IBA concentration seems to be 1 mg/l for all the three clones and the trend of root length for clones on media with different levels of IBA except the control was not significant (Table 3). Munguatosha et al. (2013) on their study for the effect of auxin and cytokinin on plant regeneration of the closely related Musacea family banana genotypes reported that there were increasing trend of root length with increasing concentration of IBA up to 2 mg/L which is not in conformity with this study due to species difference.

Plantlets cleaned from Xanthomonas pathogen by shoot tip culture technique

The result of plant extract sample culture on YPSA showed that mixed bacterial and fungal colonies on the media within 18 to 24 h of incubation under 27°C temperature set incubator (figure 5).

The colony growth was in different manner than positive control sample culture of *Xanthomonas compestris* pv. musacearum in which bacterial colony was grown after 48 h. Colony growth was also evident on media inoculated by suspension sample prepared from control sample plantlets regenerated from non-inoculated suckers of the three clones. There was no any microbial colony growth on the control petridishes with sterile distilled water stricked on media even after four days of inoculation. The colony growth pattern in this study is different from that of previous report by Eshetu (1981) in that it was very faster and mixed colonies together with fungus in the present experiment than in previous report that distinct colonies appear after 48 h incubation at 25 to 28°C on YPSA medium.

Bermita et al. (2004) in their genetic variability and biotechnological studies for the conservation and improvement of *Ensete ventricosum* reported that microorganisms that influence the micro propagation efficiency are also associated with field-grown enset. The microbes recorded were regarded as endophytic since they were not prevented by standard surface sterilization procedures or by addition of antimicrobial compounds in the medium. Similarly, in this study microbial colony growth was observed on YPSA media from sample suspension prepared from healthy *in vitro* growing plantlets including shoots regenerated from control clean plantlets not artificially inoculated with pathogen. To confirm weather the bacterial colonies were *Xanthomonas* pathogen or not, pathogenecity test was done on healthy growing suckers of the most susceptible clone three months Arkiya suckers. Any of the plants inoculated did not show disease symptom. In addition to different colony, growth pattern and pathogenecity test result of plantlets *in vitro* and in acclimatization are growing healthy which indicates that 100% of the plantlets regenerated from diseased mother plants were cleaned from *Xanthomonas compestris* pv. *musacearum*.

Acclimatization

Among the plantlets acclimatized in pots filled with sterile soil mix, 90% survival was recorded for Digomerza plantlets (Figure 6B) and 80% for both Arkiya and Mazia after 15 days of acclimatization. Whereas, 60% survival was recorded for Digomerza plantlets acclimatized to soil mix without sterilization followed by 50% for Mazia. Soil sterilization has high effect on survival of plantlets and Digomerza clone has high performance in terms of survival in acclimatization even in non sterile acclimatization media. Rajani (2006) reported 80% survival rate of ginger *in vitro* plantlets acclimatized to solar sterilized peat soil mix after 15 days of acclimatization which is in agreement with the present finding. Dawit (2009) also reported an average (70%) survival rate for cassava *in vitro* regenerated plantlets acclimatized under screen house to a sterile soil mixture of red soil, compost and sand in the ratio of 1:1:2 respectively.

In conclusion, the protocol developed for *in vitro* regeneration of disease free plantlets of enset for three clones is efficient and new in its type. Where surface sterilizing explants with 70% of ethanol for 5 min followed by 2% of chlorox for 10 and 20 min were found as optimum. MS media with 4.5 mg/L BAP and 1.5 mg/L NAA in combination is sufficient for shoot induction and growth, whereas, 1 mg/L IBA is obtained to be optimum for root induction and growth.

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Table 1. Effect of plant growth regulators (BAP and NA) combination on different clones for shoot induction and growth parameters of the three clones.

Hormone combination		Clone	Days to single shoot initiation	Days to multiple shoot induction	Number of shoots	Shoot length (cm)	Shoot fresh weight(g)	Shoot dry weight (g)
BAP (mg/L)	NAA (mg/L)							
0	0	Mazia	9.00^{a}	23.00^{b}	1.33^{g}	0.65^{f}	0.08^{g}	0.02^{g}
0	0	Digomerza	8.33^{b}	23.33^{ab}	1.67^{fg}	0.69^{f}	0.07^{g}	0.023^{g}
0	0	Arkiya	8.25^{b}	25.33^{a}	2.33^{fg}	0.61^{f}	0.08^{g}	0.020^{g}
1.5	0.5	Mazia	8.67^{ab}	18.66^{c}	2.67^{fg}	4.53^{c}	0.17^{e}	0.07^{f}
1.5	0.5	Digomerza	6.67^{c}	17.00^{cd}	3.33^{f}	2.83^{d}	0.16^{e}	0.09^{ef}
1.5	0.5	Arkiya	6.67^{c}	14.00^{efg}	1.66^{fg}	3.17^{d}	0.17^{e}	0.11^{de}
3	1	Mazia	6.00^{ed}	15.67^{de}	19.00^{b}	7.33^{a}	0.45^{cd}	0.098^{ef}
3	1	Digomerza	5.50^{ef}	15.00^{def}	15.00^{d}	8.06^{a}	0.46^{cd}	0.15^{cd}
3	1	Arkiya	5.75^{ed}	15.67^{de}	5.33^{e}	4.66^{bc}	0.48^{bc}	0.16^{c}
4.5	1.5	Mazia	5.50^{ef}	11.66^{h}	23.00^{a}	7.46^{a}	0.55^{b}	0.20^{b}
4.5	1.5	Digomerza	6.25^{dc}	14.33^{efg}	16.33^{c}	7.36^{a}	0.81^{a}	0.22^{b}
4.5	1.5	Arkiya	6.17^{dc}	13.33^{fgh}	7.00^{e}	5.40^{b}	0.41^{d}	0.28^{a}
6	2	Mazia	6.08^{ed}	12.67^{gh}	17.00^{c}	2.53^{d}	0.14^{ef}	0.11^{de}
6	2	Digomerza	5.50^{ef}	15.33^{def}	20.33^{b}	2.73^{d}	0.16^{e}	0.13^{cd}
6	2	Arkiya	5.25^{f}	14.00^{efg}	15.67^{c}	1.50^{e}	0.18^{e}	0.103^{ef}
LSD (5%)			0.59	2.03	1.89	0.75	0.07	0.033
CV (%)			5.41	7.35	11.23	11.44	13.76	16.86

Means with the same letter in the same column are non significant at 5% significance level. LSD: Least Significant Difference; CV: coefficient of variation.

Table 2. Main Effects of hormones and clones on number of leaves per shoot.

Means of hormone treatments combination main effect		
BAP (mg/L)	**NAA (mg/L)**	**Leaf number**
0	0	1.00[c]
1.5	0.5	1.89b
3	1	3.77[a]
4.5	1.5	4.11[a]
6	2	1.77[b]
LSD (5%)		0.48

Clones main effect	
Clones	**Leaf number**
Mazia	2.80[a]
Digomerza	2.66[a]
Arkiya	2.06[b]
LSD (5%)	0.36
CV (%)	13.03

Means with the same letter in the same column are non-significant at 5% significance level. CV: Coefficient of variation; LSD: least significant difference.

Table 3. Effect of IBA on root induction and growth related parameters of Mazia, Digomerza and Arkiya clone shoots proliferated.

IBA (mg/L)	Clone	Days to root induction	Mean length of roots	Root fresh weight/plant (g)	Root dry weight/plant (g)
0	Mazia	15.33[ab]	2.50[i]	0.019[h]	0.007[j]
0	Digomerza	16.50[a]	4.33[de]	0.018[h]	0.006[j]
0	Arkiya	15.33[ab]	3.26[h]	0.018[h]	0.004[j]
0.5	Mazia	14.66[bc]	5.03[b]	0.034[g]	0.011[i]
0.5	Digomerza	14.00[bced]	5.96[a]	0.035[g]	0.014[h]
0.5	Arkiya	12.83[def]	4.32[de]	0.037[g]	0.015[h]
1	Mazia	13.50[cde]	4.60[cd]	0.044f	0.020[g]
1	Digomerza	14.17[bcd]	4.96[b]	0.043[f]	0.023[f]
1	Arkiya	12.66[ef]	4.46[de]	0.050[e]	0.03[e]
1.5	Mazia	10.50[g]	4.16[e]	0.053[de]	0.033[cd]
1.5	Digomerza	11.83[fg]	4.90[bc]	0.055[cd]	0.036[b]
1.5	Arkiya	13.33[cde]	3.63[fg]	0.061[b]	0.032[d]
2	Mazia	14.00[be]	3.76[f]	0.074[a]	0.038[a]
2	Digomerza	12.83[def]	4.80[bc]	0.064[b]	0.035[b]
2	Arkiya	13.66[cde]	3.43[gh]	0.057[c]	0.034[bc]
LSD (5%)		1.37	0.17	0.0039	0.0019
CV (%)		5.99	2.40	5.26	4.89

Means with same letter in a column are non-significant at 5% significance level. LSD: Least significant difference; CV: coefficient of variation.

Table 4. Main effect of hormone concentration on number of roots per shoot.

Mean of hormone treatment main effect	
IBA	**Number of roots/shoot**
0	1.44[d]
0.5	2.55[c]
1	3.55[ab]
1.5	3.11[b]
2	3.77[a]
LSD (5%)	0.51
CV (%)	15.51

LSD: Least significant difference.

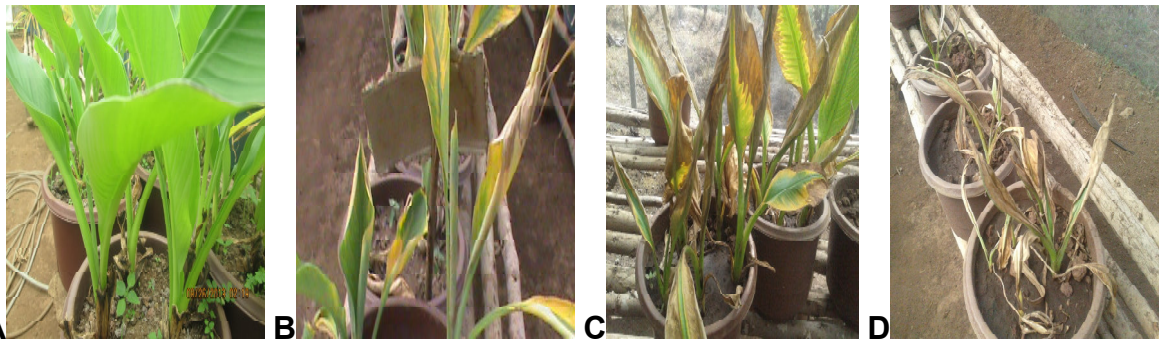

Figure 1. Mother plants used as explants source: A. Clean suckers of Enset before artificial inoculation, B. Diseased Mazia suckers, C. Diseased Digomerza suckers, D. Diseased Arkiya suckers.

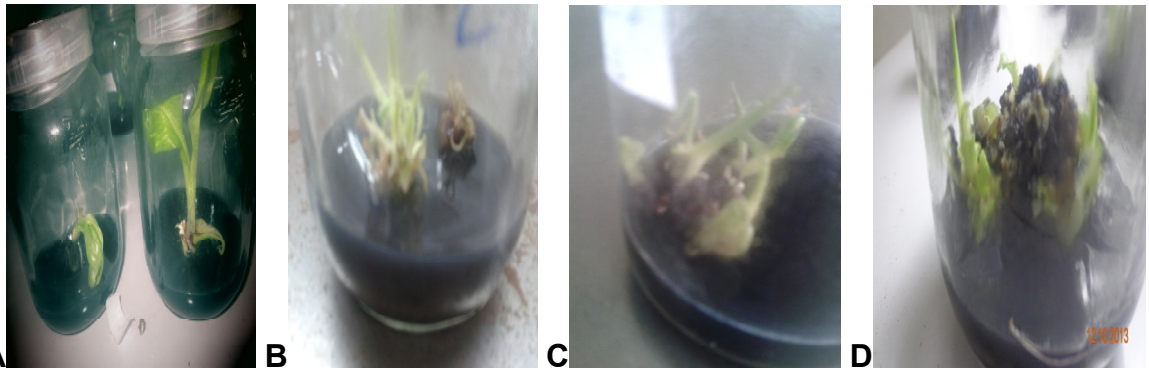

Figure 2. Shoot induction and growth at 4.5 mg/L BAP and 1.5 mg/L NAA. A. Initiated Mazia explants, B. Multiple shoots from Arkiya explants, C. Multiple shoots from Digomerza explants and D. Multiple shoots from Mazia explants.

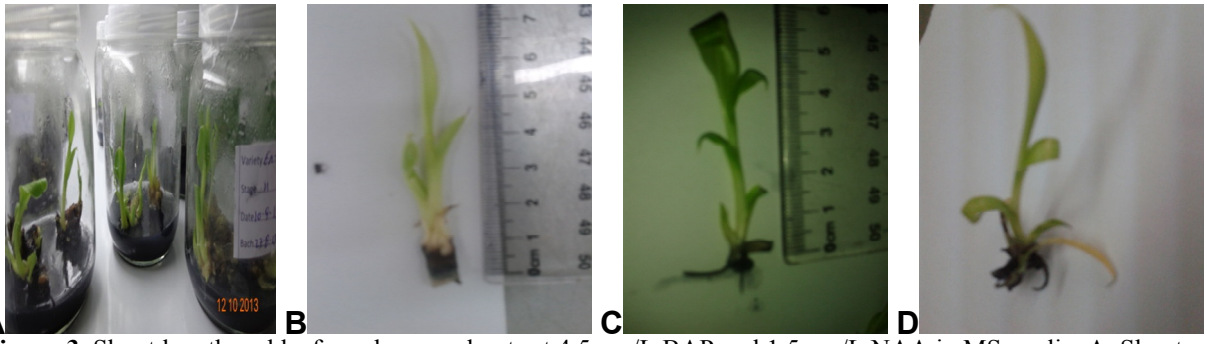

Figure 3. Shoot length and leaf number per shoots at 4.5 mg/L BAP and 1.5 mg/L NAA in MS media. A. Shoots of Mazia on elongation, B. Shoots of Digomerza, C. Shoots from Mazia explants, D. Shoots from Arkiya.

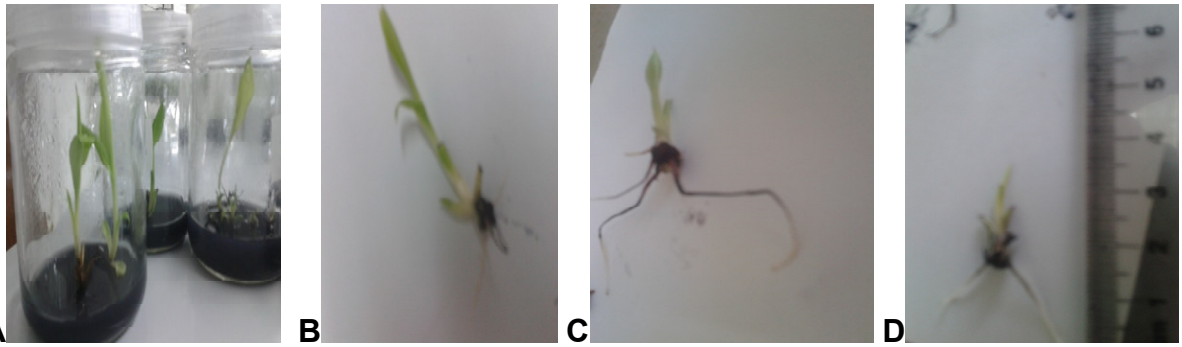

Figure 1. Plantlets in rooting MS media with 1mg/l IBA. A. Regenerated plantlets of Mazia. B. Mazia shoots with roots, C. Roots of Digomerza clone, D. Roots of Arkiya clone.

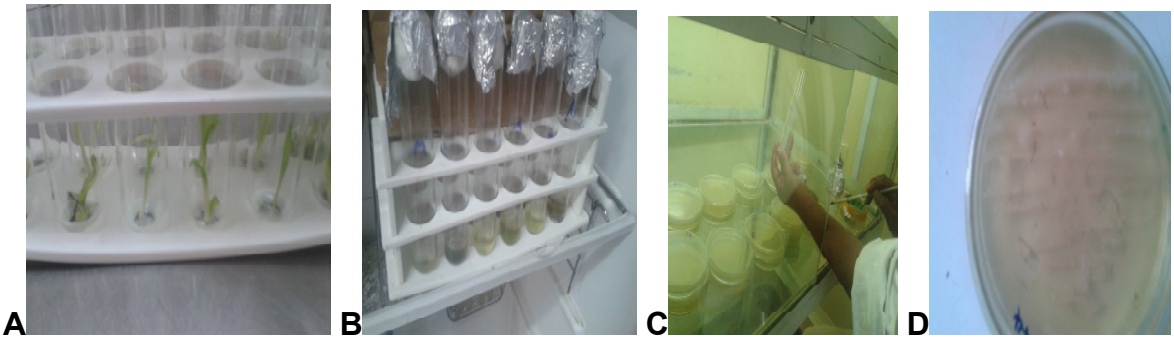

Figure 2. Procedures followed for pathogen testing. A. Shoots of the three clones for suspension sample preparation, B. suspension sample prepared from the shoots, C. Inoculation of samples to YPSA media under laminar hood. D. Mixed colony grown on YPSA.

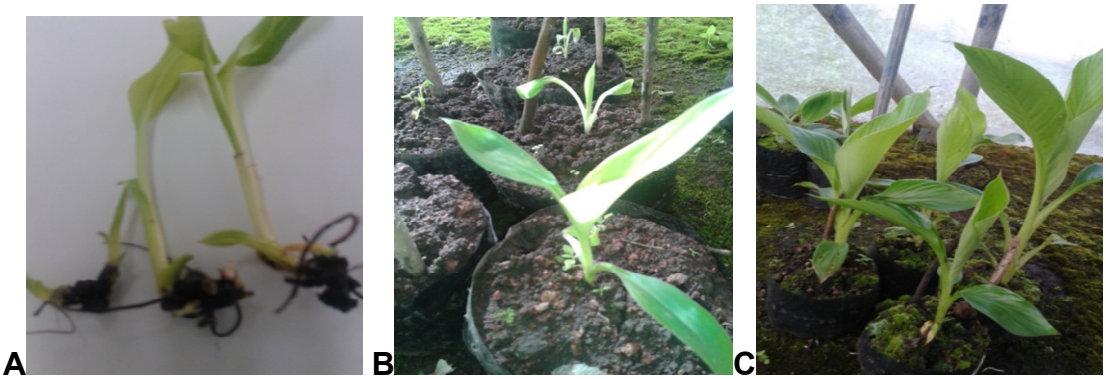

Figure 3. Plantlets acclimatized to soil mix of red ash and loam soil. A. Plantlets ready for acclimatization, B. Acclimatized plantlets after 15 days. C. Acclimatized plantlets current status.