academicJournals

Vol. 14(2), pp. 108-118, 14 January, 2015
DOI: 10.5897/AJB2013.12008
Article Number: 4D1516149619
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Micropropagation of *Phytolacca dodecandra* L'Herit (Endod var. E-44)

Jiregna Daksa[1], Balcha Abera[1]* and Tewodros Taddese[2]

[1]Department of Biology, College of Natural Sciences, Jimma University, Jimma 378, Ethiopia.
[2]Jimma Agricultural Research Center, Ethiopian Institute of Agricultural Research, Ethiopia.

Received 10 January, 2013; Accepted 5 November, 2014

***Phytolacca dodecandra* L'Heit (Endod var. E-44) is a medicinal plant of high saponin content used as a snail control agent in schitosomiasis. The aim of this study was to develop a micrpropagation protocol for *P. dodecandra*. Nodal and shoot tip explants were sterilized and cultured on MS medium supplemented with different types of concentrations and combinations of auxin and cytokinin for shoot induction, multiplication and root formation. Sodium hypochlorite at a concentration of 1.56% exposure of 12 min gave high percentages (81.67%) of survived explants for nodal followed by 1.3% concentration of NaOCl exposure of 10 min (73%) for shoot tip. 6-Benzylaminopurine (2.22 µM) was found to be an optimum concentration for shoot induction, yielding 80% for nodal and 70% for shoot tip explants. The combination of BAP (2.22 µM) with kinetin (2.33 µM) was obtained as optimum concentration yielding 13.4 and 11.03 shoots per explants for nodal and shoot tip, respectively for shoot multiplication. Half - strength MS medium with IBA (2.45 µM) and IAA (2.85 µM) yielded more than 90% root formation with optimum root number and length. Sterilized soil mix of 2:1:1 (top forest soil: coffee husk: sand) was optimized yielding 80% on transparent polyethylene plastic box. This developed micropopagation protocol is highly significant for large scale propagation of this species as a source of saponin extraction for drug development against endemic schitosomiasis.**

Key words: Explants, E-44 cultivar, plant growth regulators, micropropagation, detergents.

INTRODUCTION

Phytolacca dodecandra L'Herit belongs to family Phytolacaceae commonly known as soapberry; a climbing plant with hanging branches, growing up to 10 m and usually fruits twice a year. Endod is a dioecious plant with small berries which, when dried, powdered and stirred in water, yield a foaming detergent solution that has been traditionally used for washing clothes in Ethiopia, Zambia, and other African countries (Demeke et al., 1990; Polhill, 2000). *P. dodecandra* has remained the prime candidate for use as a snail control agent in schistosomiasis control programmes (Molgaard et al., 2000), which is endemic in 76 countries of Africa, Asia and Latin America affecting millions of people (Erko et al., 2002). Endod possess molluscicidal compound saponin (Lemmatoxin) linked with glucose and/or galactose molecules synthesized in endod berries. Endod variety E-

*Corresponding author. E-mail: balcha_abera@yahoo.com. Tel: 02510917023409.

Figure 1. Mother stockplant of *P. dodecanda* established from seedlings and cuttings under greenhouse condition during experiment, Jimma Agriculture Research Centere.

44 was selected and recommended for further researches for its high composition (25%) of saponins (Lemma et al., 1972; Parkhurst et al., 1974; Legesse, 1993; Lee et al., 1998).

Endod can be propagated by seed, but the germination percentage is very low (20%) (Demeke et al., 1990). Vegetative propagation by stem cuttings has been attempted, but very few percentages were achieved (Demeke and Hagus, 1990). Besides, the major constraints in the cultivation of endod through the conventional propagation method are its low productivity due to poor genetic stock, incidence of pests and diseases and its latency. Moreover, Endod being a dioecious plant the female flowers open later when the male flowers gets ready, so that there will be incompatibility of pollination and fertilization (Lee et al., 1998; Zimudzi, 2007).

Micropropagation protocol development and other molecular studies were made for some Phytolaccaceae particularly *P. dodecandra* (E-3 and E-17) ((Bonnes et al., 1992; Koch et al., 1996; Castellar et al., 2011) but no micropropagation protocol was developed for the E-44 cultivar of endod. Therefore, as there is no protocol that has been developed for endod (*P. dodecandra*) particularly for E-44, micropropagation technique is essential to achieve rapid mass multiplication and distribution of improved variety as well as disease free plantlets of this most important plant.

The main purpose of this study was, therefore, to develop a micropropagation protocol for selected endod (*P. dodecandra*) cv. E-44 from shoot tip and nodal explants; and the five specific objectives of this study were to determine: (1) concentration and duration of exposure of endod explants to sodium hypochlorite (NaOCl) for surface sterilization; (2) effect of different BAP concentration on culture initiation; (3) effect of BAP alone and in combination with kinetin on shoot multiplication; (4) effect of different IAA, IBA and NAA concentrations on root induction; and (5) survival percentages of plantlets after acclimatization.

MATERIALS AND METHODS

Plant material

Seedlings of endod variety (cv. E-44) were obtained from Root Crops Research Division, Bishoftu Agricultural Research Center (BARC) 48 km east of Addis Ababa. The plant cuttings were also collected from Institute of Pathobiology (IP), Addis Ababa University (AU) and planted on sterilized soil that has a combination of top forest soil, coffee husk and sand (1:2:1) at greenhouse condition of JARC. Both seedlings and cuttings were then grown and kept as mother stockplant under Greenhouse conditions of Jimma Agricultural Research Center (JARC) at average temperature (25 ± 2°C) and relative humidity (RH). The mother stockplant was daily watered with tap water and also treated with 0.3% Mancozeb at 15 days interval to control fungal infection. Very young, health and vigorous type of the plant was particularly treated to be used as a source of explants. The overall experiment (July 29, 2011 to April 30, 2012) was conducted at Plant Biotechnology Laboratory of Jimma Agricultural Research Center (JARC) 353 km Southwest of Addis Ababa, Ethiopia.

Explants and surface disinfection

Shoot tip and nodal (2^{nd} and 3^{rd} from the top interchangeably) segment explants (1.5 - 2.0 cm) were collected from healthy and vigorously growing mother stockplants (Figure 1). The excised explant materials were washed three to five times with tap water and detergents and kept in 0.3% manchozeb solution for 20 min. This was transferred to the laminar flow hood cabinet, rinsed repeatedly with sterilized distilled water and surface sterilized in 70% of ethanol for 30 s and at different concentration of sodium hypochlorite (5.25% NaOCl) for 5', 7', 10', 12' and 15' time of exposure then rinsed with sterilized distilled water three to four times to remove the residual effect of these sterilants. To facilitate the reaction two drops of 1 mg/1 ml of Tween - 20 was added into all the sterilant solutions prior to treatment. The explants were kept in L - Cysteine - HCl (0.25 g/l) solution for 15 min. Then the treated explants were rinsed repeatedly four times with sterilized distilled water and kept in for 10 min in this sterilized distilled water under aseptic condition. This is also to remove the residual portion of the sterilants from the body of explants. Finally the effect of time exposure was determined for both explants from the culture between two weeks intervals.

Growth regulators stock preparation

Different growth regulators were prepared by weighing and dissolving the powder in distilled water at the ratio of 1 mg/ml. To begin the dissolving process three to four drops of 1 N NaOH or 1 N HCl were added based on the specific requirement of the growth regulators. Then, the volume was adjusted by adding distilled water. Finally, growth regulators' stock solutions were stored in a refrigerator at a specific temperature for each growth regulator.

Culture medium preparation

Culture medium was prepared by taking the proper amount of Murashige and Skoog (1962) stock solutions (mg/l). Full-strength of MS with 3% of sucrose as carbohydrate source (w/v) for shoot initiation and multiplication was added at a pH of 5.8 whereas, half-strength of MS with the same pH were used for root induction. Agar (7 g l^{-1}) (Sigma, St Louis MO, USA) was added after the volume and pH of the medium was adjusted. The media was sterilized by autoclaving at a temperature of 121°C with a pressure of 15PSi for 15 min and stored at room temperature.

Culture initiation

Shoot tip and nodal segment explants were inoculated in test tubes containing full MS (Murashige and Skoog, 1962) media supplemented with BAP (2.22, 4.44, 8.88 and 22.20 µM) sucrose (30 g l^{-1}), and agar (7 gl^{-1}) (Sigma, St Louis MO, USA). Free growth hormone MS medium was used as a control. One explant per test tube was cultured with 10 test tubes per treatment. The cultured test tubes were incubated in the light condition (1500 - 2000 lux) in growth room for three weeks. Then, clean and shoot inducing explants were transferred to fresh media and incubated for another three weeks under light conditions.

The same experiment was repeated three times. Data on the percentage of initiated shoots per explants were scored after three weeks of the second passages of the culture. The averages of initiated percentages were received from each replicates for four different BAP concentrations. So there were 4X2 factorial combinations in Completely Randomized Design (CRD), to test the four BAP concentrations against the two explants, where 0 is used as a control.

Shoot regeneration

All healthy initiated shoots were transferred to free growth hormone medium of half - MS length for four days to reduce latent effects of the previous medium composition. Highly initiated shoots were transferred to shoot multiplication MS fresh medium supplemented with BAP (2.22, 4.44, 6.66 and 8.8 µM) and kinetin (2.33, 4.65, 6.98 and 9.3 µM) alone or in combination with each other during fourth week having free PGRs as a control. There were five replicates with five explants per jars for each was cultured in each medium. The same treatments were repeated two times to reduce the number of errors. The cultures were maintained at 25 ± 2°C with a 16 h photoperiod at a light intensity of 1500 - 2000 lux from cool white florescent 60 watt bulbs. After four weeks of culture on shoot elongation and multiplication media, the average height of elongated shoots and average number of shoots per explants were recorded**.** The same procedure was repeated for the same treatment for accuracy of the data. Therefore, the experiment was 4x4x2 factorial combinations in CRD and treatment with free PGRs as control.

Root formation

The regenerated shoots with minimum average length of 2 cm were cultured on half - strength MS media supplemented with 15 g/l of sucrose and IBA (1.23, 2.45, 4.9 and 7.35 µM) for root induction. Similarly the effect of IAA and NAA were evaluated with the same concentration given for IBA. Half strength of MS was used as control. Therefore, the experiment was 4x3x2 factorial combinations in CRD having 0 as a control. There were five replicates with five shoots cultured for each jars. The same procedures for the same experiments were repeated twice for the accuracy of the data. The average percentage of root forming shoots per explant from each jars of the treatments and the average number and length of roots per shoot was scored after three weeks of the culture. To reduce the residual latent effect of the previous experiments of medium composition the plantlets were stayed on free PGRs half-strength of MS medium for four days before culturing on rooting medium.

Acclimatization

After the plantlets were elongated and roots are initiated, they were considered to be ready for acclimatization *ex vitro*. Therefore, individual plantlets were removed from the jars and washed carefully under warm but not hot water to facilitate the removal of agar from their roots. Then, they were transferred to sterilized potting mix of forest soil: well decomposed coffee husk: sand, at a respective v/v ratio of 2:1:1 in transparent polystyrene plastic boxes of 25 x 16 x 7 cm dimension. They were then kept in a transparent polyethylene tunnel covered by 70% shade net above it on Iron farmed table. The system was designed to give high relative humidity (80 - 90%) to prevent desiccation for ten days. Afterwards the plantlets were transferred to a 70% RH. In the tunnel, the water was sprayed everyday twice without creating water logging and meant to maintain relative humidity (RH) as high as possible. Starting from the 15^{th} day, the RH within the system was reduced to gradually to 60% at the end of the month. After the month, the plantlets were transferred to a 70% shade net, where they were retained for a month. Later they were transferred to a 30% shade net and maintained there for another month. The survival percentages of plantlets were registered in each step.

Statistical analysis

The experiment was laid in Completely Randomized Deign (CRD) with factorial treatment combinations. The experimental design was set to 4 × 2, 4 × 4 × 2 and 4 × 3 × 2 factorials for culture initiation, shoot regeneration and rooting, respectively. Treatment was one factor and explants were another factor. Each shoot tip and nodal culture in a jar was the observational unit. There were five replications per treatment and five explants per for each replicate. All explants were cultured on a free PGRs medium prior to their use for an experiment; so as to avoid any sort of carryover effects from previous culture medium they were retained. Data collected from each experiment was subjected to statistical analyses using the SAS statistical software (version 9.2) and ANOVA was constructed, followed by mean separation using Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ) at α=5% which was estimated for grouping significant factors on variables from non-significant one. Means with the same letters in a column that are not significantly different from each other were considered by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ) at α=5%.

RESULTS

Explants and surface disinfection

The concentration of sodium hypochlorite, duration of explants' exposure to sterilant and interaction of sodium hypochlorite with time had highly significant effect ($p<0.0001$ at α=5%) on the level of contamination and plant tissue death. There was also highly significant effect ($p<0.0001$ at α=5%) between the two explants. Table 1 shows that the survival of explants either from surface sterilization or microbial contamination, the highest

Table 1. Interaction effect of Sodium hypochlorite concentrations and its time of exposure on sterilization of explants.

NaOCl (%)	Time (min)	Survived explants (%)	
		Nodal (Mean ± SD)	Shoot tip (Mean ± SD)
1.3	5	24.00 ± 3.46^{j}	26.00 ± 1.00^{e}
1.65	5	30.00 ± 0.00^{ij}	26.67 ± 1.15^{e}
2.08	5	32.00 ± 0.00^{hij}	34.00 ± 2.64^{e}
2.6	5	45.00 ± 2.64^{cdefg}	39.67 ± 0.58^{e}
1.3	7	37.67 ± 3.79^{efgh}	54.33 ± 2.31^{e}
1.65	7	38.67 ± 2.52^{fghi}	54.00 ± 1.00^{bc}
2.08	7	40.67 ± 2.52^{ghi}	47.67 ± 4.72^{cd}
2.6	7	46.00 ± 2.64^{cdef}	34.67 ± 1.15^{e}
1.3	10	57.33 ± 2.08^{b}	73.00 ± 1.73^{a}
1.65	10	53.00 ± 1.00^{bc}	63.33 ± 7.23^{b}
2.08	10	45.33 ± 1.15^{cdefg}	49.00 ± 6.24^{cd}
2.6	10	43.00 ± 2.64^{cde}	33.00 ± 1.73^{e}
1.3	12	76.00 ± 6.08^{a}	72.00 ± 6.08^{a}
1.65	12	81.67 ± 2.52^{a}	59.67 ± 5.51^{b}
2.08	12	60.33 ± 7.77^{b}	44.00 ± 1.00^{d}
2.6	12	43.33 ± 0.58^{edfg}	34.33 ± 2.08^{e}
1.3	15	59.33 ± 4.16^{b}	45.67 ± 3.78^{cd}
1.65	15	49.00 ± 1.00^{cd}	47.33 ± 3.05^{cd}
2.08	15	47.67 ± 2.08^{cde}	33.33 ± 0.58^{e}
2.6	15	38.00 ± 1.73^{fghi}	25.33 ± 3.21^{e}

Means with the same letters in a column are not significantly different from each other by Ryan - Einot - Gabriel - Welsch multiple range test (REGWQ) at α=5%.

percentage was achieved from NaOCl (1. 65% v/v) for 12 min after keeping in 70% of alcohol for 30 s. For nodal culture 81.67± 2.52% of explants survived from the nodal explants and ready to be transferred to the next subculture. This was followed by 76.00± 6.08% of clean explants at 12 min and 1.3% of sodium hypochlorite for nodal explant source. The highest percentages (73.00 ± 1.73) was achieved from shoot tip explant at 10 min exposure and 1.3% of sodium hypochlorite; and then followed with 72.00 ± 6.08% at a time of 12 min and 1.3% of sodium hypochlorite. The lowest concentration of sodium hypochlorite at 5 and 7 min resulted to low percentages of survived explants for both nodal and shoot tip explants. Among the given concentrations of sodium hypochlorite the highest concentrations (2.08 and 2.6% of sodium hypochlorite) at longer time (12 and 15 min) of exposure gave low percentages of survived plantlets (Figure 2).

Effect of BAP on shoot induction

Clean plantlets from surface sterilization were transferred to shoot induction medium that contains BAP (2.22, 4.44, 8.88 and 22.2 µM) and start to respond after seven to fourteen days. The mean separation of the interaction of explants by BAP on percent shoot induction is shown in Appendix II. The responses include high callus formation, swelling on the upper and lower part of the cultured explants, color change and direct shoot induction (Figure 4). For both explants, after being cultured on growth initiation medium for three weeks the frequency of shoot induction percentages were differ at different BAP concentration.

Analysis of variance showed that, there was highly significant effect ($p<0.0001$ at $\alpha = 5\%$) among the interaction of different BAP concentration on percent shoot initiation for both explants (Appendix II and III). ANOVA also revealed that there was no significant effect ($P< 0.0803$ at $\alpha = 5\%$) in interaction of BAP with explants (BAP*explant). But among the explants, there is highly a significant effect ($p< 0.0001$ at $\alpha = 5\%$). Figure 3 shows that high percentage (80.00 ± 3.56%) shoots were induced from nodal explants at BAP concentration of 2.22 µM followed by 73.25 ± 3.30% on a medium with 4.44 µM of BAP. In the case of shoot tip explants, high percentages of shoots were induced at 70.00 ± 4.97% followed by 66.75 ± 2.36% at 2.22 µM and 4.44 µM of BAP, respectively (Figure 3 and Appendix II). From the given concentrations, high concentrations of BAP and a medium without BAP resulted in low percentage of shoots induction. The lowest percentage induction was observed from 22.2 µM of BAP for both nodal and shoots tip, 42 and 35%, respectively.

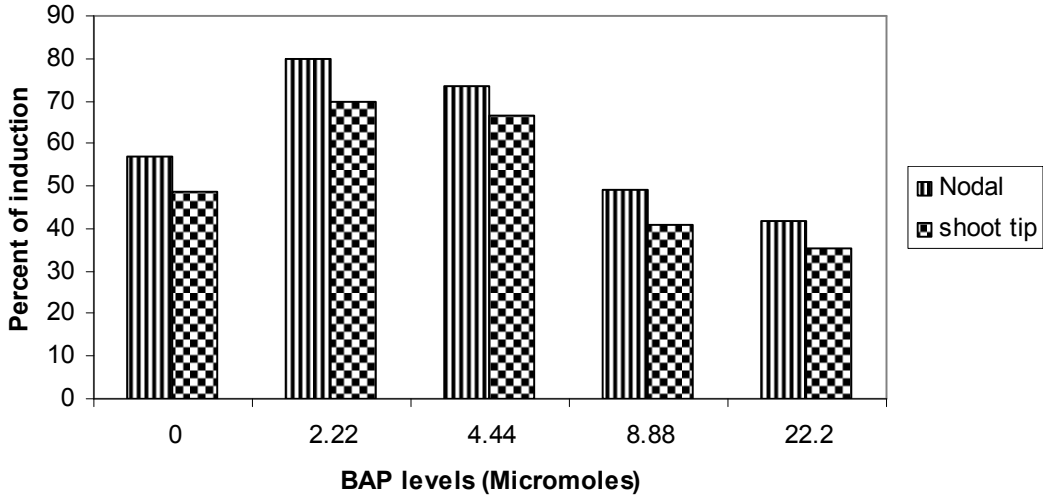

Figure 2. The effect of BAP at different levels on nodal and shoot tip explants induction of *P. dodecandra.*

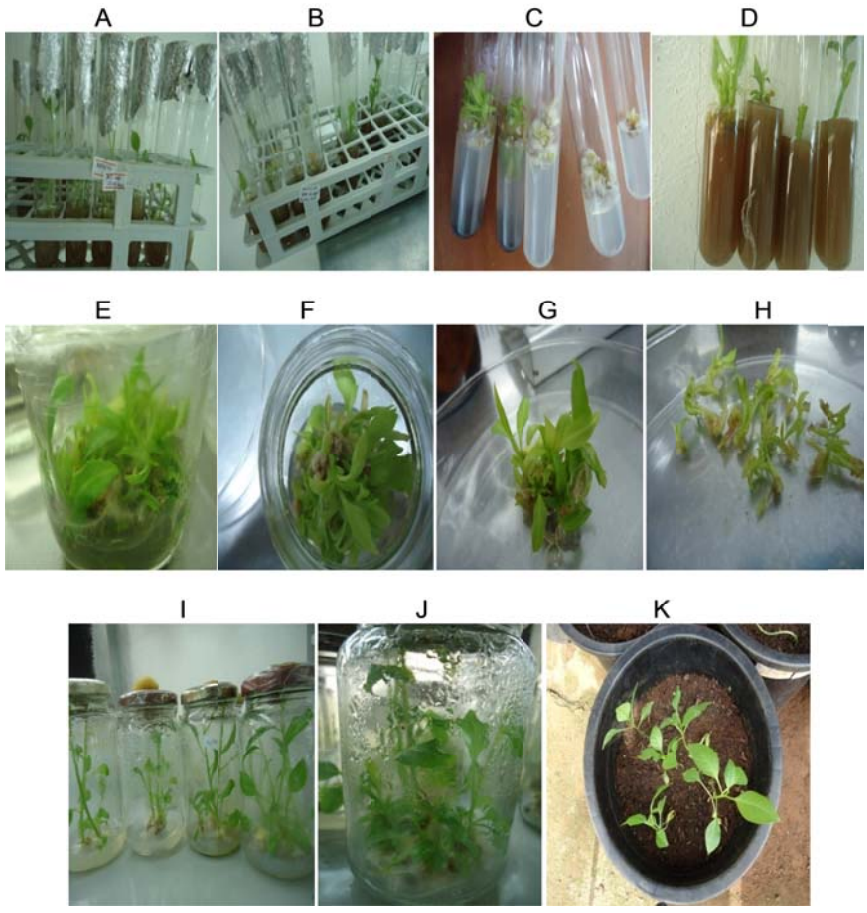

Figure 3. Micropropagation of *Phytolacca dodecandra*. (A) Culture from nodal explants at optimum concentration and exposure time**. (B)** Culture from shoot tip at optimum concentration and exposure of time. (**C)** Shoot induction on 2.22 µM of BAP with $AgNO_3$. **(D)** Shoots regenerated on 2.22 µM of BAP and 2.33 µM kinetin. **(F)** Shoots regenerated on 6.66 µM of BAP. (G, H) plantlets from a single explant from 6.66 µM of BAP. **(I, J)** Rooting on half - strength medium with 2.45 µM IBA. **(K)** Plantlets on black polyethylene pot.

Effects of BAP and Kinetin on shoot multiplication

Contamination free, vigorous and highly induced plantlets were transferred to a medium containing BAP (2.22, 4.44, 6.66 and 8.88 µM) in combination with kinetin (2.33, 4.65, 6.98 and 23.26 µM) or alone for four weeks (Table 2).

ANOVA showed that there was highly significant effect ($p<0.0001$ at $\alpha= 5\%$) between each growth hormones at different concentration (Appendix IV). It also showed that there was highly significant effect on shoot number multiplication rate between the two explants ($p<0.0001$ at $\alpha= 5\%$). In the case of shoot length, the interaction effect of BAP and kinetin as well as this this plant growth regulators with explants had highly significant effect ($p<0.0001$ at $\alpha= 5\%$) whereas, there had no significant effect ($p<0.8053$ at $\alpha= 5\%$) between each explants (Tables 2 and 3).

High multiplication rate was obtained at the combination of BAP (2.22 µM) and kinetin (2.33 µM) for both nodal (13.40 ± 0.60) and shoot tip explants (11.03 ± 0.55). This was followed by 6.66 µM of BAP which resulted to shoot number of 10.13 ± 0.81 for nodal and 8.73 ± 0.2 for shoot tip explants. A medium without growth regulators (BAP and kinetin) and a medium with high concentration of BAP and kinetin alone or in combination of the two resulted to low multiplication rate (Table 2). This was for both explants at different concentration and with different shoot number per explants.

Longest average shoot length was obtained from 6.66 µM of BAP alone and 4.65 µM of kinetin alone that is the same with that of BAP alone (3.27 ± 0.30 cm) in the case of nodal explants. The shoot length that was obtained from shoot tip was slightly longer than the shoot height observed from nodal explants. The longest shoot height was gained from 4.65 µM of kinetin alone, 3.80 ± 0.20 cm. Medium with high multiplication rate per explants gave slightly smaller average length of shoot when compared to a medium giving more number of shoot per explants.

Effects of IAA, IBA and NAA on root proliferation

The plantlets with minimum average length of 2 cm from multiplication medium were cultured on rooting medium on different concentration of IAA, IBA and NAA. Root induction was observed after a week and the optimum root number and root length were obtained after three week (Figure 3). ANOVA shows that auxins (IAA, IBA and NAA) concentration had highly significant effect ($p < 0.0001$ at $\alpha= 5\%$) on rooting percentages, root number and root height (Appendix V), but there was no significant effect on IBA concentrations on shoot elongation.

Percentage increases/decrease in shoot height, root number and root height per explants is given by plus or minus (±). Means within a column followed by the same letters are not statically significant at $\alpha = 5\%$ by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

Table 4 shows higher rooting percentage (99.33 ± 1.15%) on half - strength MS medium at 2.85 µM of IAA followed by 92.33 ± 3.05% of rooting at 2.45 µM of IBA the same medium with minimum callusing. Among the given concentrations, the root inducing auxin hormones high concentration resulted to less rooting percentages with greater diameter of callus induction. Naphthalene acetic acid (NAA) at a concentration of 8.06 µM resulted to less percentage of rooting (40.33 ± 1.53%) that was less than the root induced (64.00 ± 3.61%) on half strength medium without any plant growth regulators.

Regenerated shoots with average height of two to four centimeters were sub-cultured on a medium prepared for rooting purpose on half - strength MS medium of the different auxins at different concentrations. The longest shoots were observed from a medium of 2.45 µM of IBA (8.00 ± 0.50 cm) followed by 1.23 µM of IBA (7.90 ± 0.36 cm). Smallest shoot height where observed from 1.34 µM of NAA (4.50 ± 0.30 cm) followed by 5.37 µM of NAA (5.33 ± 0.76 cm).

Largest mean number of roots were counted (14.23 ± 0.45) from 2.45 µM of IBA followed by (11.67 ± 0.35) on 2.86 µM IAA. Highest concentrations of auxins resulted in less number of root. Relatively, less number of roots were observed (Table 4) from NAA at 8.06 µM (3.27 ± 0.31).

Longest root height was observed from a medium responding less number of shoots. There was smaller number of roots on IAA and IBA at different concentrations that accounted larger number of roots than NAA (Figure 3).

Acclimatization

The best plantlets from rooting after three weeks were planted for acclimatization both on transparent polystyrene plastic boxes and black polyethylene pot with dark shade nets. Different soil mixes were sterilized and tested as a best soil type for endod from the soil combination of top forest soil, compost (coffee husk) and sand (Appendix VI). For the first week plantlets on a pot were wilted and only average of 45% were survived. The best soil mix, in which high percentages (82.2) of plantlets survived and developed was on top soil, coffee husk and sand (2:1:1) respectively (Figure 3).

DISCUSSION

Microbial contamination is one of the most serious problems in plant cell and tissue culture. These contaminants may be introduced with explants into the culture medium and affect the potential of the plants for regeneration. Disinfectants such as alcohol, sodium hypochlorite (NaOCl) and Silver nitrate (AgNO3) with few drops of Tween 20 hamper the growth rate of fungus and bacteria on the growth medium (Odutayo et al., 2007; Ryan, 2008).

Table 2. Effects of BAP and Kinetin on the multiplication rate and shoot length per explants for in vitro propagation of endod.

Level of PGRs		Nodal		Shoot tip	
BAP (µM)	Kinetin (µM)	Shoot number (mean ± SD)	Shoot length (mean ± SD) (cm)	Shoot number (mean ± SD)	Shoot length (mean±SD)(cm)
-	-	2.67 ± 0.58^{kl}	3.00 ± 0.00^{abcd}	2.53 ± 0.42^{l}	3.00 ± 0.20^{bcd}
2.22	-	4.00 ± 0.20^{hij}	2.40 ± 0.40^{cde}	3.13 ± 0.23^{k}	2.50 ± 0.10^{def}
4.44	-	4.80 ± 0.20^{f}	3.07 ± 0.50^{abcd}	4.80 ± 0.20^{f}	3.07 ± 0.50^{bcd}
6.66	-	10.13 ± 0.81^{a}	3.27 ± 0.30^{a}	8.73 ± 0.21^{b}	2.77 ± 0.25^{bcde}
8.88	-	4.53 ± 0.42^{fg}	2.53 ± 0.11^{abcde}	4.37 ± 0.15^{fg}	2.20 ± 0.20^{ef}
22.20	-	3.27 ± 0.31^{k}	2.80 ± 0.20^{abcde}	2.16 ± 0.29^{l}	2.77 ± 0.20^{bcde}
-	2.33	5.27 ± 0.31^{ef}	2.13 ± 0.23^{e}	3.87 ± 0.31^{kij}	2.57 ± 0.21^{cdef}
-	4.65	4.20 ± 0.20^{ghij}	3.27 ± 0.12^{a}	4.20 ± 0.20^{ghij}	3.80 ± 0.20^{a}
-	6.98	3.77 ± 0.25^{hij}	2.80 ± 0.20^{abcde}	4.80 ± 0.35^{f}	3.37 ± 0.15^{ab}
-	9.3	3.20 ± 0.20^{k}	2.33 ± 0.12^{de}	3.60 ± 0.20^{hij}	2.87 ± 0.12^{bcd}
2.22	2.33	13.40 ± 0.60^{a}	2.43 ± 0.21^{bcde}	11.03 ± 0.55^{a}	2.70 ± 0.26^{cdef}
2.22	4.65	7.67 ± 0.58^{c}	2.90 ± 0.36^{abcd}	7.33 ± 0.42^{c}	2.57 ± 0.21^{cdef}
2.22	6.98	7.33 ± 0.58^{c}	2.63 ± 0.32^{abcde}	4.73 ± 0.31^{f}	2.93 ± 0.12^{bcd}
2.22	9.3	3.87 ± 0.23^{hij}	3.00 ± 0.00^{abcd}	4.47 ± 0.42^{fgh}	2.93 ± 0.12^{bcd}
4.44	2.33	6.33 ± 0.58^{d}	3.00 ± 0.00^{abcd}	3.00 ± 0.00^{k}	3.00 ± 0.00^{bcd}
4.44	4.65	8.93 ± 0.31^{b}	2.83 ± 0.29^{abcde}	6.33 ± 0.58^{d}	3.20 ± 0.20^{bc}
4.44	6.98	7.00 ± 0.00^{c}	2.53 ± 0.23^{abcde}	8.50 ± 0.50^{b}	2.20 ± 0.20^{ef}
4.44	9.3	3.80 ± 0.72^{hij}	2.83 ± 0.15^{abcde}	4.67 ± 0.58^{fg}	2.70 ± 0.26^{cdef}
6.66	2.33	8.00 ± 0.40^{bc}	2.67 ± 0.12^{abcde}	4.73 ± 0.31^{f}	2.10 ± 0.17^{f}
6.66	4.65	5.33 ± 0.58^{ef}	2.43 ± 0.21^{bcde}	6.47 ± 0.42^{d}	2.67 ± 0.29^{bcde}
6.66	6.98	6.07 ± 0.11^{d}	2.53 ± 0.23^{abcde}	7.00 ± 0.40^{c}	2.17 ± 0.29^{ef}
6.66	9.3	3.93 ± 0.12^{hij}	2.47 ± 0.31^{bcde}	3.33 ± 0.58^{k}	2.47 ± 0.30^{def}
8.88	2.33	7.83 ± 0.76^{bc}	3.13 ± 0.12^{abcd}	7.50 ± 0.50^{c}	2.87 ± 0.23^{bcd}
8.88	4.65	6.73 ± 0.25^{d}	2.60 ± 0.20^{abcde}	6.67 ± 0.58^{d}	3.00 ± 0.20^{bcd}
8.88	6.98	5.50 ± 0.50^{e}	2.90 ± 0.17^{abcd}	4.97 ± 0.55^{f}	2.50 ± 0.10^{def}
8.88	9.3	4.13 ± 0.31^{ghij}	3.17 ± 0.35^{abcd}	4.13 ± 0.42^{ghij}	2.87 ± 0.23^{bcd}
CV % = 7.62					

Means within a column followed by the same letters are not statically significant at α= 5% by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

Table 3. *R yan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ)* for shoot number and shoot height multiplying potential of nodal and shoot tip explants.

Explants	Shoot Number means	Pr > |T|	Shoot Length means (cm)	Pr > |T|
Nodal	5.84^{a}		2.76^{c}	0.8053
Shoot Tip	5.29^{b}	<.0001	2.75^{c}	

Means within a column followed by the same letters are not statically significant at α= 5% by Ryan - Einot - Gabriel - Welsch Multiple Range Test (REGWQ).

Plant material collection and handling were done according to Zerihun et al. (2009). The collected explants were gently washed with tap water and detergents, followed by keeping in antifungal (0.3% Mancozeb) before treating with four different concentrations of NaOCl (1.3, 1.65, 2.08 and 2.56%) were used in this study. According to Demeke et al. (1990), using NaOCl is best sterilant for endod. ANOVA revealed statistically high significant effect ($p < 0.0001$ at $\alpha = 5\%$) among sodium hypochlorite concentrations, time of exposures, explants, the interaction of sodium hypochlorite concentration and time of exposure (time*NaOCl) and interaction of the three factors (time*NaOCl*explant). This showed that long and short time exposure matters for the

Table 4. Effect of various concentrations of IAA, IBA and NAA on rooting of proliferated shoots of *P. dodecandra* cultured on half - strength MS medium

Level of PGRs (µM)			Rooting percentages	Shoot height (cm)	Root number	Root height (cm)
IAA	IBA	NAA	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)
0	0	0	64.00 ± 3.61^{e}	6.00 ± 1. 00^{b}	3.83 ± 0.29^{fg}	4.17 ± 0.29^{a}
0	0	1.34	73.67 ± 1.53^{d}	4.50 ± 0.30^{c}	5.43 ± 0.60^{e}	2.83 ± 0.29^{cbd}
0	0	2.68	84.00 ± 2.00^{c}	6.50 ± 0.50^{b}	7.93 ± 0.83^{d}	2.37 ± 0.15^{de}
0	0	5.37	49.67 ± 3.51^{f}	5.33 ± 0.76^{bc}	4.97 ± 0.21^{ef}	3.07 ± 0.40^{bc}
0	0	8.06	40.33 ± 1.53^{g}	6.73 ± 0.50^{ab}	3.27 ± 0.31^{g}	3.87 ± 0.11^{b}
0	1.23	0	81.33 ± 3.21^{c}	5.40 ± 0.53^{bc}	9.33 ± 0.61^{c}	2.47 ± 0.30^{cde}
0	2.45	0	92.33 ± 3.05^{b}	6.47 ± 0.42^{b}	14.23 ± 0.45^{a}	2.77 ± 0.25^{cbd}
0	4.9	0	71.00 ± 1.00^{d}	6.23 ± 0.40^{b}	9.80 ± 0.53^{c}	2.50 ± 0.10^{cde}
0	7.35	0	42.33 ± 2.52^{g}	5.77 ± 0.25^{bc}	5.83 ± 0.29^{e}	1.90 ± 0.10^{e}
1.45	0	0	85.00 ± 3.00^{c}	7.90 ± 0.36^{a}	9.97 ± 0.55^{c}	2.90 ± 0.10^{cbd}
2.85	0	0	99.33 ± 1.15^{a}	8.00 ± 0.50^{a}	11.67 ± 0.35^{b}	3.23 ± 0.38^{b}
5.71	0	0	85.33 ± 1.53^{c}	6.37 ± 0.32^{b}	10.00 ± 0.50^{c}	1.87 ± 0.12^{e}
8.57	0	0	69.33 ± 2.52^{de}	6.03 ± 0.45^{b}	9.00 ± 0.40^{c}	1.97 ± 0.25^{e}
Mean			72.13	6.24	8.10	2.76
CV %			3.44	8.36	5.99	8.83
R - square			0.98	0.82	0.99	0.92

survival percentage of the explants to the given concentrations (1.3, 1.65, 2.08 and 2.56%) of sodium hypochlorite. Observation of higher contamination rate of explants in treatment combinations having lower concentration of sodium hypochlorite and short time of exposure might be due to insufficient concentration and time of treatment to kill the contaminating microbes. The higher the concentration of sodium hypochlorite with longer time of exposure, the better the removal of microbial contaminants, which was obtained as a synergistic effect of chlorine concentration and duration of time of treatment. The active ingredient of the chlorine, which is a powerful oxidant in high concentrations, can kill bacteria, fungi etc., inhibiting of bacterial cell wall synthesis, blocking bacterial DNA replication, bacterial protein synthesis and altering their shape and the physiological activities of their enzymes (Reed and Tanprasert, 1995; Hartmann et al., 2002); but, this high concentration yield fewer percentages of plantlets. This might be due to inability of the plant tissue to survive with high concentration of sterilant, causing plant tissue death (Ervin et al., 2002). This plant tissue death was highly observed in shoot tip explant. The immaturity of the soft tissues of the shoot tip might cause inability to resist high concentration of sterilants than nodal explants (Hartmann et al., 2002).

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied within a medium and the growth substances induced endogenously (Hartmann et al., 2002). 6-Benzylamino purine is the best cytokinin growth hormone used for shoot induction in plant tissue culture (Singh et al., 2007; Sugla et al., 2007) and the same is true for *P. dodecandra* tissue culture (Omer et al., 1995; Demeke et al., 2007).

The present study also used several concentrations of BAP and obtained 80% from nodal and 70% from shoot tip explants induced at 2.22 and 4.44 µM of BAP, respectively. High concentration of BAP induced lesser percent (42%) of shoot and resulted stunt shoot growth. Demeke et al. (2007) reported in their preliminary experiment as 0.44 µM of BAP induced 1.9 shoots per explant on E-17 variety. The present study showed that 2.22 µM BAP induced an average of 3.13 shoots from shoot tip explants and 4 shoots from nodal explants. The possible differences of this study (Demeke et al., 2007) might be due to the genotype and the BAP concentrations used. The significance effect of BAP concentration with interaction of explants might be due to the concentration effect of BAP concentration. Once both explants pass the sterilization stages there was no significant effect among these explants in shoot induction.

Callus induction at the base of the explants was the major problem in this study. The medium prepared with silver nitrate (1% $AgNO_3$) reduced unnecessary induction of callus and results to more number, green and vigorous shoots. Such problems were found in tissue culture of plants with high endogenous auxin concentrations. This might be due to the stress/wounding of the explants during subculture which possibly induced ethylene biosynthesis. This ethylene activity results to callus formation (Mayor et al., 2003; Wu et al., 2006; Anantasaram and Konchanapoom, 2008). Thus, Silver nitrate has proved to

be a very potent inhibitor of ethylene action, its application gives a good shoot induction and multiplication with minimum callus formation (Kumar et al., 2007). The effect of silver nitrate is not only the reduction of callus formation but also reducing the plantlets from microbial loads, because silver nitrate is also used as antibiotics for this purpose (Orlikowska., 1997; Ozudogru et al., 2004).

Shoot multiplication and development increased with increasing the concentration of cytokinins (BAP and kinetin) up to 6.66 µM or when in combination less than this. High concentration induced rapid but stunt growth and little number of shoots. In the present study 2.22 µM of BAP with 2.33 µM Kinetin gave maximum number of shoots than highly concentrated cytokinins. From the study of Demeke and Hagus (1992) 4.7 shoots per explants for nodal explants on MS medium with 0.44 µM BAP and 0.27 µM of GA_3 were reported. For shoot tip explants also 3.1 on MS medium with 0.44 µM of BAP for other varieties (E-3 and E-17) were reported.

In the present study, a mean of 13.4 numbers of shoots for nodal explants and 11.03 shoots for shoot tip explants were recorded at 2.22 µM of BAP and 2.33 µM of kinetin combination. At very high concentration of either BAP or Kinetin or in combination of the two, shoots number per explant was fewer than even MS medium free of plant growth hormones.

The study of Demeke and Hagus (1992), Omer et al. (1995) also revealed that concentration of plant growth regulators had significant effect for shoot multiplication and elongation on micropropagation of other varieties of *P. dodecandra*. They also reported that there was no significant effect in shoot multiplication and shoot number among the explants. The present study also revealed highly significant effect between treatments (BAP*kinetin), between treatments and explants (treatment*explants) for shoot multiplication and elongation, whereas there was no significant effect among explants for shoot length.

The same to shoot induction, callus induction at the base of the explant was the major problem for shoot multiplication and growth. In this study, the medium prepared with $AgNO_3$ (100 mg/l) gave optimum shoot number and shoot length per explants, which is similar to the obser9ation of (Ozudogru et al., 2004).

Plantlets observed from this medium were more vigorous, green, more number of plantlets compared to the multiplications on medium free of $AgNO_3$. The same observation was reported using silver nitrate on some tissue cultures of plants having the same callus induction problems (Wu et al., 2006; Anantasaram and Konchanapoom, 2008).

Small concentrations and even optimized concentrations of cytokines for multiplication had induced the roots that also inhibit shoot multiplication. Either using small concentration or addition of any auxin hormones at a level of any concentration showed the same effect. This showed that there is endogenous auxin concentration (Daffalla et al., 2011).

For any micropropagation protocol, successful rooting of microshoots is a prerequisite to facilitate their establishment in soil. An addition of an auxin, mainly IAA, IBA and NAA to the medium or free growth hormone was essential to induce rooting in the regenerated shoots (Hartmann et al., 2002; Daffalla et al., 2011). In this study, half - strength MS medium supplemented with IAA (1.45, 2.85, 5.71 and 8.57 µM), IBA (1.23, 2.45, 4.9 and 7.35 µM) and NAA (1.34, 2.68, 5.37 and 8.06) were evaluated and relatively 2.85, 2.45 and 2.68 for IAA, IBA and NAA respectively gave good rooting percentage. The concentrations beyond these led to a decrease in the number of roots and root length per rooted explant and rooting rate. The root elongation phase is very sensitive to auxin concentration, and it is inhibited by high concentration of auxin in the rooting medium. Daffalla et al. (2011) reported that roots may require a less concentration of auxin to grow, but root growth is strongly inhibited by its higher level because at this level, auxin induces the production of ethylene, a root growth inhibitor.

In present study, compact callus formation inhibiting the growing shoots and very small number and inadequate root length were observed at the higher concentration of almost all the given auxins. This result is in agreement with the study of Demeke and Hagus (1992) who reported that increasing concentration of auxins resulted greater production of callus with short, thick and small number of roots. They also reported the production of thin and long roots with minimum callus induction from low concentration of auxins. Their result also confirmed IBA at low concentration gave 90% of root induction with minimum calli induction for the other varieties, whereas, 92.33% of root induction for variety E-44 type. However, the present study resulted in high percentage (99%) of root induction by IAA at 2.86 µM.

Analysis of variance for the present study revealed that there was highly significance effect among the concentrations of the given auxins for all experimental variables, namely, rooting percentages, root number, and root height except insignificancy of IBA on shoot elongation. The increase or decrease in concentration of these hormones led variability of root induction percentage. Induction percentage, less at high concentration; shoot elongation that was longer at low concentration; number of roots, high at optimum low concentration; and root length that was also based on the auxins concentrations. The longest root was measured from free PGRs half-MS length medium. What worth mentioning and should not be passed without mentioning is the rooting medium was also used as shoot elongation. So there is no need of developing another protocol for shoot elongation.

In vitro induced shoots are very delicate and prone to sudden environmental changes that may damage the plant unless it is gradually adapted to the new environment.

Hence, rooting and acclimatization is required to enable shoots adapt to the natural environment. The essentiality of appropriate root development *in vitro* is for successful establishment of endod shoots during acclimatization. For this case, green and vigorous plantlets with large number of roots and root length of greater than 2cm were planted in transparent polyethylene plastic box. The best soil mix for endod acclimatization was 2:1:1 (top soil, coffee husk and sand).

The plantlets were planted on either polyethylene coffee box that was made to provide good aeration, whereby more percentages of plantlets were survived and a plastic pot covered with black shed net. Due to unsuitability of the environment, for the first week plantlets were wilted and only 45% of plantlets were survived from plastic box. In a Polyethylene coffee box and on sterilized 2:1:1 (top forest soil, coffee husk and sand) soil mixes, 82.2% of the plantlets were survived.

Conclusion

Surface sterilization process is very important to minimize and avoid the risk of culture loss due to microbial contamination. Highly concentrated sterilants give free of microbial contamination but less number of survived plantlets due to synergetic effect to the plant tissue. So that optimum sterilants' concentration and exposure of time is crucial for micropropagation of *P. dodecandra* variety (cv. E-44).

Increasing concentration of BAP in shoot induction gives less percent of shoots induced. So, the best level of BAP concentration is 2.22 µM for both nodal and shoots tip explants. For mass propagation of E-44, multiplication protocol development is indispen-sible. To do this BAP and kinetin combination of the two at low concentration (2.22 µM) for both nodal and shoot tip were optimized in this study. Root induction and unnecessary callus formation occurs almost at all stages, shoot induction, multiplication and rooting stages without using any auxin hormone. This indicates that naturally the plant has high concentration of endogenous auxin. Thus, it was difficult to manage the ethylene biosynthesis that occurs due to high concentration auxins and that results growth retardation, inhibition of shoot induction and multiplication and even rooting.

Silver nitrate was used in this study to inhibit ethylene biosynthesis, and then callus induction was highly reduced. This results high percentages of shoot induction and large number of shoots when prepared with shoot induction medium and multiplication medium, respec-tively, but promising result was not occurred in rooting medium. The culture was stayed as it was cultured without induction of roots. From this we can conclude, Silver nitrate also inhibits auxins, thus the amount of auxins required in the medium cannot influence for root induction.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge Jimma Agricultural Research Center (JARC) for providing laboratory, chemicals and relevant facilities, Jimma University (JU) for financial support, Bishoftu Agriculture Research Institute (BARI) and Institute of Pathobiology (AAU) for plant material/germplasm permission.

REFERENCES

Bonnes MS, Mabry TJ (1992). Tissue - culture of endod (*Phytolacca dodecandra* L.herit) - growth and production of ribosome - inactivating proteins. Plant Cell Rep. 11:66-70.

Castellar A, Gagliardi RF, Mansur E (2011). *In vitro* propagation and establishment of callus and cell suspension cultures of *Petiveria alliacea* L., a valuable medicinal plant. J. Med. Plant. Res. 5:1113-1124.

Daffalla HH, Abdellatef E, Elhadi, EA, Khalafalla MM (2011). Effect of growth regulators on *in vitro* morphogenic response of *Boscia senegalensis* (Pers.) Lam. Poir. using mature zygotic embryos explants. Biotech. Res. Int. 10:1-8

Demeke T, Hughes HG (1990). Micropropagation of *Phytolacca dodecandra* through shot - tip and nodal, cultures. Plant Cell Reports 9:390-392.

Demeke T, Hughes HG, Lee CW (1992). Propagation of *Phytolacca dodecandra* (endod) by stem cuttings and seed. Trop. Agricul. 69:301-303.

Erko B F, Abebe N, Berhe G, Medhin T, Gebre - Michael T, Gemechu T, Gundersen SG (2002). Control of *Schistosoma mansoni* by the soapberry endod (*Phytolacca dodecandra*) in Wollo, Northeastern Ethiopia: post - intervention prevalence. East. Afr. Med. J. 79:198-201.

Ervin GN, Wetzel RG (2002). Effects of Sodium hypochlorite sterilization and dry cold storage on germination of *Juncus effuse* L. Wetlands 22:191-195.

Hartmann HT, Kester DE, Davies FT, Geneve RL (2002). Plant tissue culture **In**: *Plant Propagation, principles and practices*, 7th ed. PHI Learning, New Delhi, India.

Hughes I, (2002). Phytolacca N.F. (Poke Root) **In**: *Potters cyclopedia of botanical drugs and preparations*. Herbedla, News zealand.

Koch PE, Bonness MS, Lu H, Mabry JJ (1996). Protoplasts from *Phytolacca dodecandra* L'Herit (endod) and *P. americana* L. (pokeweed**)**. Plant Cell Reports 15:824-828.

Kumar V, Parvatam G, Ravishankar GA (2009). Silver nitrate ($AgNO_3$) - a potential regulator of ethylene activity and plant growth modulator. Electr. J. Biotech. 12:1-6.

Lee HH, Lemma A, Bennett JJ (1998). The Use of endod *(Phytolacca dodecandra)* to control the zebra mussel *(Dreissena polymorpha).* Lewis, USA.

Legesse W, (1993). Endod, a potential natural pesticide for use in developing countries http://collections.infocollections.org/ukedu/en/d/Jid22ie/6.4.html#Jid22ie.6.4 Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia. Assessed on April 3, 2011.

Lemma A, Brody G, Newell GW, Parkhurst RM, Skinner WA (1972). Studies on the molluscicidal properties of endod (*Phytolacca dodecandra*): Increased potency with butanol extraction. J. Parasitol. 58:104-107.

Mayor ML, Nestares G, Zorzoli R, Picardi LA (2003). Reduction of hyperhydricity in sunflower tissue culture**.** Plant Cell, Tissue and Organ Culture 72:99-103.

Mølgaard P, Chihaka A, Lemmich E, Furu P, Windberg C, Ingerslev F, Halling B (2000). Biodegradability of the molluscicidal saponins of

Phytolacca dodecandra. Reg. Toxicol. Pharma. 32:248-255.
Murashige T, and Skoog F (1962). A revised medium for rapid growth and bioassay with obacco issue cultures''. Physiol. Plantrum, 15:473-497.
Odutayo OI, Amusa NA, Okutade OO, Ogunsanwo YR (2007). Sources of microbial contamination in tissue culture laboratories in southwestern Nigeria. Afr. Agri. Res. 2:067-072
Omer EA, El - Menshawi BS, Edris M, Abou-Hadid AF (1995). Micropropagation of *Phytolacca dodecandra* L'herit. ISHS Acta Horticulturae 434: strategies for market oriented greenhouse poduction, Egypt.
Orlikowska T, (1997). Silver nitrate inhibits bacterial growth in plant tissue cultures. Agri. cell Report 29:25-32.
Ozudogru EA, Tokatli YO, Akcin A (2004). Effect of silver nitrate on multiple shoot formation of Virginia - type peanut through shoot tip culture. *In vitro* cell. Develop. Bio.41:151-156.
Parkhurst RM, Thomas DV, Skinner WA, Cary LW (1974). Molluscicidal saponins of *Phytolacca dodecandra:* Lemmatoxin. Can. J. Chem. 52:702-705.
Polhill RM (2000). Phytolaccaceae **In**: Edwards, S., Tadesse, M. Demissew, S. and Hedberg, I. (ed.). Flora of Ethiopia and Eritrea. *Magnoliaceae to Flacourtiaceae*. The National Herbarium, Addis Ababa University, Addis Ababa, Ethiopia and Department of Systematic Botany, Uppsala University, Uppsala, Sweden.
Reed BM, Tanprasert P (1995). Detection and control of bacterial contaminats of plant tissue cultures: A review of recent litratures. Plant Tissue Cult. Biotechnol. 1:37-43.
Ryan J (2008). Understanding and managing cell culture contamination, Technical Bulletin, 900 Chelmsford St. Lewell, MA.
Singh BS, Singh MP (2007). Findamental of plant biotechnology. Satish serial puplication house, New Delhi, India.
Sugla T, Purkayastha J, Singh SK, Solleti SK, Sahoo L (2007). Micropropagation of *Pongamia pinnata* through enhanced axillary branching. In vitro cell Dev. Biol. Plant. 43:409 - 414.
Wu LM, Wei YM, Zheng YL (2006). Effects of silver nitrate on the tissue culture of immature wheat embryos. Russian Journal of Plant Physiology 53:530-534
Zerihun A, Ayelign M, Alemayehu T, Wondyfraw T (2009). Efficient *in vitro* multiplication protocol for *Vanilla planifolia* using nodal explants in Ethiopia. Afr. J. Biotech. 8:6817-6821.
Zimudzi C (2007). *Phytolacca dodecandra* L'Hér. Record from Protabase. Schmelzer, G.H. and Gurib - Fakim, A. (Editors). PROTA (Plant Resources of Tropical Africa), Wageningen, Netherlands. < http://database.prota.org/search.htm> assessed on April 11, 2011.