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In vitro regeneration protocol through direct organogenesis for *Jatropha curcas* L. (Euphorbiaceae) accessions in Ethiopia

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***Jatropha curcas* L. is among important tree crops in the world with a potential for biofuel production. In Ethiopia, there is a soaring investors' interest to produce Jatropha in the country for biodiesel production. However, insufficient good quality propagation material is a major production constraint. A study was undertaken to establish a protocol for *in vitro* mass propagation of three Ethiopian Jatropha accessions viz. Metema, Adami Tulu and Shewa Robit through direct organogenesis from nodal explant. The result revealed that the highest percentage of shoot induction (86-90%) was achieved on MS medium with BAP (1 mg/L) and IBA (0.5 mg/L) for all the three accessions. The maximum number of shoots (6) was obtained for Metema when BAP (0.5 mg/L) with Kn (0.5 mg/L) was used. Whereas, the maximum (3.2 cm) shoot length was recorded for Shewa Robit on media with 0.5 mg/L Kn. The highest rooting percentage (84.8-88%) and maximum root number (5.43) were recorded on media supplemented with 0.25 mg/L IBA. Shewa Robit and Metema had longer roots on media with 0.25 mg/L IBA. Finally, the plantlets were successfully established in greenhouse with survival rate of 86.67% for Shewa Robit followed by 73.33 and 66.67% for Metema and Adami Tulu, respectively. This study provided optimal protocol for micro-propagation of Jatropha accessions through direct organogenesis to boost its production.**

Key words: *Jatropha curcas*, biofuel, organogenesis, plant growth regulators.

INTRODUCTION

Jatropha (*Jatropha curcas* L.) is a succulent shrub or small tree, which belongs to the large Euphorbiaceae family. It originated from Central America. From the Caribbean, Jatropha was probably distributed by Portuguese seafarers via the Cape Verde Islands and former Portuguese Guinea (now Guinea Bissau) to other countries in Africa and Asia in the 16th century (Heller, 1996; BAZ, 2007). It is currently found worldwide in most tropical countries including Ethiopia (Mekuria et al., 2008).

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Jatropha is a multi-purpose plant which has been exploited for various purposes such as soil erosion control, firewood, hedges, green manure and traditional medicines (Carels, 2013). On the other hand, the seed oil of Jatropha is also used as soap manufacturing ingredient, paints and as a biodiesel to substitute kerosene (Kumar and Sharma, 2008). Among many other attributes and importance of Jatropha, in recent years it has gotten special attention for being a priority feed stock in production of biodiesel.

Biodiesel is an alternative diesel fuel made from different types of renewable sources such as plant oils and animal fats. It is environmentally friendly fuel with low emission profiles and also non-toxic and biodegradable (Abdulla et al., 2011). Biofuels derived from non-edible oils such as Jatropha (*J. curcas*), Mahua (*Madhuka indica*), Cardoon (*Cynara cardunculus*), Paradise tree (*Simarouba glauca*), Castor (*Ricinus communis*), Karanj (*Pongamia pinnata*) are more economical and suitable compared to edible oils (Naresh et al., 2012). In some of the developed nations (US and European countries) edible oils such as rape seed and soybean oils are used for biodiesel conversion. However, in developing countries usage of edible oils for biofuels is not feasible (Knothe, 2000).

Among the plant species producing raw materials for biofuels, Jatropha is one of the plant species that stimulates the highest interest in tropical and subtropical regions. It has been identified as most suitable oil seed bearing plant due to its various favorable attributes like high oil content, hardy nature, adaptability in a wide range agro-climatic conditions, need for less irrigation and less agricultural inputs, pest resistance, short gestation periods and suitable traits for easy harvesting (Heller, 1996; Edrisi et al., 2015).

In Ethiopia, Jatropha grows in various parts of the country such as in Wolayita, Metekel, Southern Wollo, Northern and Eastern Shoa, Tigray, Gamo Gofa zones and Gambella region (Getinet et al., 2009). Traditionally, farmers in Ethiopia mainly used Jatropha plants as living fences and as a structural means of conserving soil and water (Zufan, 2010). Now, in connection with green economy goals, the Ethiopian government has begun to promote supply of fuels from locally produced biofuel without affecting food self-sufficiency and by reducing environmental impacts (FDRE, 2007, 2011). The strategy intends to make the country carbon neutral by 2030 (FDRE, 2011). To achieve these goals, biofuels which can be produced from non-edible oil like Jatropha is the best solution.

Based on this strategy several local and foreign private investors have started growing plants for producing biodiesel. There are about 85 companies registered in Ethiopia to invest in biofuels, mainly Jatropha (Mengistu, 2013). Most of these companies have the intention of going for large-scale commercial development (Abreham and Belay, 2015). However, several challenges remain before the plant biomass can be commercially exploited. Its supply on a large scale requires massive production of phenotypically uniform plant material of a very high quality within a short time-frame that is adapted to the growth conditions of the plantation areas. The increase in plantation area creates high demand of good planting material to be available and these calls for a means that can provide planting material in large scale and within short period of time. There is a need to establish mass multiplication technique to meet the large-scale demand and easy supply of Jatropha plant (Medza Mve1 et al., 2013; Mengistu, 2013).

Traditionally Jatropha is propagated through seed and vegetative cutting. The most common method to obtain Jatropha plantlets is by seed germination, which can be severely limited by poor seed viability, low germination percentage, inadequate rooting in growth plants in small pots and the delayed rooting of seedlings (Openshaw, 2000). Seed propagated plants are also not true to type and can result in oil concentration variations between 8-54% (Ovando-Medina et al., 2011).

Vegetative propagation of Jatropha through stem cuttings has been achieved however the established plants are not deep rooted and hence, they easily get uprooted when cultivated in lands with poor top soil (Openshaw, 2000). Despite their profuse vegetative growth, the number of seeds produced per plant is very low and the seeds show a low seed fecundity, which is reduced by 50% within 15 months (Ginwal et al., 2004). Plants propagated by cuttings also show a lower longevity and possess a lower drought and disease resistance than those propagated by seeds (Sujatha et al., 2005). Again this conventional propagation technique has its own draw back since planting materials are one of sources for disease transmission from place to place (Genene, 2014). Besides, for an effective large scale commercialized production of Jatropha maintaining true to type genotypes, producing disease free planting materials and high number of propagule *in vitro* culture has a paramount importance. Therefore, to improve cultivation of this crop, the traditional inefficient mode of propagation should be changed and proper techniques need to be studied and put in place for mass production of the Jatropha plants.

The *in vitro* multiplication would be a useful alternative method for mass production of the plant. Micropropagation technique (Plant tissue culture) offers an opportunity for large scale production of uniform disease free planting material in a relatively short period of time and independent of the season (George, 2008). *In vitro* derived plants are frequently more vigorous and of superior quality compared to those produced by *in vivo* methods. Evaluation of tissue culture propagated plants of Jatropha revealed that they produced a better yield and yield- related traits than seed-propagated plants (Sujatha et al., 2005). That means this clonal propagation method has the advantage of producing plants that are

Table 1. Sources and growing altitudes of planting material of Jatropha accessions were used in this study.

Province (Region)	Place of collection	Altitude(m)	Collectors (seed source)
Oromia	Adami Tulu	1500	WGRC
Amhara	Metema	1000	SARC
Amhara	Shewa Robit	1250	SARC

WGRC= Wondo Genet Research Center; SARC= Sirinka Agricultural Research Center.

morphologically homogenous with an equal production potential. The culturing of plant cells or organs can overcome problems of the flowering season, pollination, pollinators, seed setting, gestation period, viral infections, etc. Besides, Jatropha is a perennial crop where flowering would mostly take more than a year in majorities of the genotypes. This would in turn hinder getting sufficient amount of seed for rapid propagation. Therefore the development of an efficient *in vitro* regeneration system would be a remarkable progress for the Jatropha business and the field of alternative energy technology (Rajore and Batra, 2007).

One of the prospective and potential ways of *in vitro* plant culture of Jatropha is organogenesis. Organogenesis refers to the process in which a unipolar structure can be derived either through differentiation of non-meristematic tissues or through pre-existing meristematic tissues (Thorpe et al., 1990; Hussain et al., 2012; Moniruzzaman et al., 2016). It is one of the widely used methods employed for *in vitro* plant regeneration. Plant regeneration through organogenesis can occur either directly or indirectly (Thorpe et al., 1990; Geetha et al., 2008).

Direct organogenesis involves the emergence of adventitious organs directly from the meristematic region of the explants without callus phase and important to ensure clonal fidelity. Several authors have regenerated Jatropha through organogenesis using different explants (Sujatha and Muktra, 1996; Sujatha et al., 2005; Rajore and Batra, 2005; Deore and Johnson, 2008; Kumar et al., 2011).

Plant regeneration from *in vitro* organogenesis is possible due to the induction of stimuli for certain metabolic pathways that will trigger changes in the pattern of cell growth and development (Almeida et al., 2012). The regulation of organogenesis *in vitro* can be achieved by different types of manipulation. These include appropriate choice of explant, age of the explant, orientation of explant, proper choice of the culture medium, plant growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light regime, temperature and humidity (Sujatha and Mukta, 1996; Sujatha et al., 2005; Feyissa et al., 2005; Deore and Johnson, 2008).

Among factors influencing *in vitro* regeneration via organogenesis, plant growth regulators in media and genotypes are the most important. The plant growth regulators (e.g., auxins and cytokinins) play an important role in organogenesis processes since their regimes can be used to manipulate the morphogenetic response of plants under in vitro cultures (Arya et al., 2009). *In vitro* plant regeneration from explants requires the presence of appropriate concentrations and combinations of plant growth regulators in the culture media (Kalimuthu et al., 2007). A sub-optimal culture medium may cause physiological disorders or death of tissue. Studies of auxins and cytokinins separately or their combinations to initiate *in vitro* organogenesis in Jatropha were reported (Sujatha et al., 2005; Deore and Johnson, 2008). In addition, genotypic differences in shoot organogenesis have been observed in a wide range of species including Jatropha. It has been reported that regeneration in Jatropha is highly genotype dependent (Kumar, 2008; Kumar and Reddy, 2010; Kumar et al., 2010; Mweu et al., 2016).

In Ethiopia, development and application of tissue culture techniques for propagation of Jatropha is at its early stage. Prior to this work, there are no enough documented studies on micropropagation of Ethiopian Jatropha accessions which can be used for mass production. Keeping in view of the importance of the crop and its propagation methods, the present study was designed to optimize *in vitro* protocol for direct organogenesis of three Ethiopian Jatropha accessions using nodal explant. Hence, the specific objectives of the study were:

(i) To optimize the concentrations and combinations of different growth regulators on MS medium for maximum proliferation of shoots from direct organogenesis;
(ii) To investigate rooting response of shoots to different IBA and NAA concentrations and combinations;
(iii) To evaluate survival rates of acclimatized plant regenerants in green house environment.

MATERIALS AND METHODS

Planting material

The seed of three Jatropha accessions were collected from Amhara and Oromia region of Ethiopia and used for these tissue culture experiments (Table 1).The seeds were germinated on growth trays containing sterilized combination of soil, sand and manure in the ratio of 2:1:1, respectively and kept in the greenhouse condition of Holeta Agricultural Research Center (HARC). They were watered thrice a week using a spraying can. After three weeks, the seed that germinated was transplanted into pot containing sterilized soil and

Figure 1. Mother stock plant of Jatropha accessions after three months.

kept as mother stock plant. After three months of growth (Figure 1); very young, health and vigorous part of the plant (nodal segment) was collected and used as a source of explants. The overall experiment (June 29, 2017 to January 30, 2018) was conducted at Plant Biotechnology Laboratory of Holeta Agricultural Research Center (HARC) 45 km West of Addis Ababa, Ethiopia.

Growth regulators stock preparation

The Plant Growth regulators (PGRs) used for the study were the cytokinin, 6-benzyl aminopurine (BAP) and Kinetin (Kn), and the auxins, indole-3- butyric acid (IBA) and α-naphthalene acetic acid(NAA). All PGRs stock solutions were prepared by weighing and dissolving the powder in distilled water at the ratio of 1 mg/ml. To begin the dissolving process, the powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH and 1N HCl based on the type of PGR (NaOH for auxins and HCl for cytokinin). Then, the volume was adjusted by adding distilled water. Finally, growth regulators' stock solutions were stored in a refrigerator at +4°C for short term use.

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 30 g/L of sucrose as carbohydrate source (w/v) for shoot initiation and multiplication were used whereas, half-strength of MS with 15 g/L sucrose were used for root induction. The pH of the medium was adjusted to 5.8 (using 1N NaOH and 1N HCl) after addition of the growth regulators. Gerlite (2.5 g/L) was added as a gelling agent 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.

Explants and surface disinfection

Nodal explant was collected from healthy and vigorously growing mother stock plants. The excised explant materials were initially rinsed under tap water for 30 min to remove the dust particles from their surface. Then the explants were treated with commercial detergent (Largo, Ethiopia) for 5 min and were rinsed well with distilled water for three to five times. Under a clean Laminar flow hood, the explants were subjected to 70% (v/v) ethanol for one minute and rinsed with sterile distilled water three to four times. Further the explant materials were then surface sterilized by 3% of Local bleach (Berekina, Ethiopia) containing two drops of Tween 20 for 15 min time of exposure. After that the explants were rinsed with sterilized double distilled water for three to four times to remove the residual effect of these sterilants. After sterilization process was completed, individual nodal segment was trimmed (l -l.5 cm) with one node and inoculated by vertical orientation on the medium.

Culture initiation

For culture initiation, single nodal explant (1-1.5 cm) was inoculated on full strength MS media supplemented with different combinations of BAP (0, 1, 1.5 and 2.0 mg/L) and IBA (0, 0.5 and 1.0 mg/L) along with Ascorbic acid (10 mg/L) for prevention of browning of cultures. Five nodal explants per culture jars and five replications for each treatment were used. The cultured explants were incubated in the light condition (1500 - 2000 lux) in growth room at 25 ± 2°C for four weeks.

Shoot multiplication and elongation

For shoot multiplication, shoots from best establishment (induction) medium were used to avoid the influence of different origin of media. Then, highly aseptically initiated shoots were transferred to shoot multiplication MS fresh medium which supplemented with various combinations of BAP (0, 0.5, 1.0, 1.5 mg/L) and Kn (0, 0.5 and 1.0 mg/L). Five shoots per culture jars and five replications for each treatment were used. 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 bulbs. After four weeks of culture, the growth response of the micro-shoots to different treatments was recorded. For shoot elongation, the multiplied shoots were transferred onto growth regulators free MS basal medium for two weeks.

Root induction

The elongated shoots with three to four leaves were excised and cultured on half strength MS media supplemented with different

Table 2. Percentage of shoot initiation from nodal explant cultures of three Jatropha accession (Metema, Adami Tulu and Shewa Robit) at different concentrations and combinations of BAP and IBA after 30 days of culture.

PGRs (mg/L)		Shoot induction (%)		
BAP	**IBA**	**Metema**	**Adami Tulu**	**Shewa Robit**
0	0	-	-	-
0	0.5	34.80 ±0.20^{op}	35.20 ±0.0.20^{o}	33.00 ±1.22^{p}
0	1.0	-	-	-
1.0	0	70.00 ±0.00^{g}	68.00 ±1.22^{h}	69.80 ±0.20^{gh}
1.0	0.5	90.00 ±0.31^{a}	86.00 ±1.00^{b}	90.00 ±0.00^{a}
1.0	1.0	24.00 ±0.00^{q}	23.14 ±0.00^{q}	25.00 ±0.5^{q}
1.5	0	74.00 ±1.00^{ef}	73.00 ±1.22^{f}	75.00 ±0.00^{e}
1.5	0.5	80.00 ±0.00^{d}	80.00 ±0.00^{d}	82.00 ±1.22^{c}
1.5	1.0	55.00 ±0.00^{k}	52.00 ±1.22^{l}	53.00 ±1.22^{l}
2.0	0	85.00 ±0.00^{b}	85.00 ±0.00^{b}	85.00 ±0.00^{b}
2.0	0.5	63.00 ±1.22^{j}	65.00 ±0.00^{i}	64.00 ±1.00^{ij}
2.0	1.0	41.00 ±1.00^{n}	43.00 ±1.22^{m}	40.00 ±0.00^{n}
CV (%)			**2.92**	
LSD (5%)			**1.86**	

Different letters (within columns and rows) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5). CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard error, n= Number of samples, '-' indicates no response.

combinations of IBA (0, 0.25, 0.5 and 1.0 mg/L) and NAA (0, 0.25 and 0.5 mg/L) for root induction. There were five replicates with five shoots cultured for each jars. Then the culture was maintained in a growth room at a temperature of 25±2°C and 16 h photoperiod provided by white florescent lamps. Each root growth parameters were recorded within four weeks of the culture.

Growth conditions

All the cultures were kept under eight hour dark period and sixteen hour photoperiod in a growth room. Artificial light was provided by parallel cool white fluorescent tubes (Philips, India) installed above the cultures. The light intensity was regulated to 1500-2000 lux and the growth room temperature was adjusted at 25 ± 2°C with relative humidity (RH) of 65-70%.

Acclimatization

After the well rooted *in vitro* propagated Jatropha plantlets are obtained, it was taken out gently from the culture vessels and the root system was washed under running tap water to remove traces of agar that prevent the absorption of nutrients from the acclimatization culture substrates by roots. A total of 45 well rooted shootlets (15 shootlets from each Jatropha accession) were transferred to plastic pots containing a mixture of sterilized sand, soil and compost in the ratio of 1:2:1, respectively and then transferred to the greenhouse for hardening. The potted plants were maintained in a greenhouse at a temperature of 25 ± 2°C with 60-75% relative humidity. The pots were covered with transparent plastic bags with random holes for air circulation and the underside of the pots was drilled for drainage. Then they were watered using sprayer every day. Plastic cover were removed partially after a week and completely removed after two weeks. Finally, after about one month, percent of plantlets successfully hardened were calculated.

Experimental design and statistical analysis

The experiment was laid out in Completely Randomized Design (CRD) for all the treatments. The experiment was comprised of different combination and concentrations of plant growth regulators combined with three accessions of Jatropha. Growth regulators were one factor and accessions were another factor. Each treatment had five replicates of culture Jars and set as experimental unit. 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 Fisher's Least Significance Difference (LSD) at α=5%.

RESULTS AND DISCUSSION

Effects of BAP and IBA on Shoot Induction

Analysis of variance showed that, there was highly significant difference (P<0.01) among growth regulator concentrations and combinations (BAP and IBA) and their interaction with the three Jatropha accession on percentage of shoot initiation. ANOVA also revealed that there was no significant difference (P>0.05) among the Jatropha accessions on percentage of shoot induction (Table 2).

The highest percentage (90%) shoot induction was recorded for both Shewa Robit and Metema accessions on MS media supplemented with combination of 1 mg/L BAP and 0.5 mg/L IBA followed by 86% for Adami Tulu accession on the same hormone combinations and concentrations. Whereas, the lowest percentage (22-25%) induction was observed from the media containing

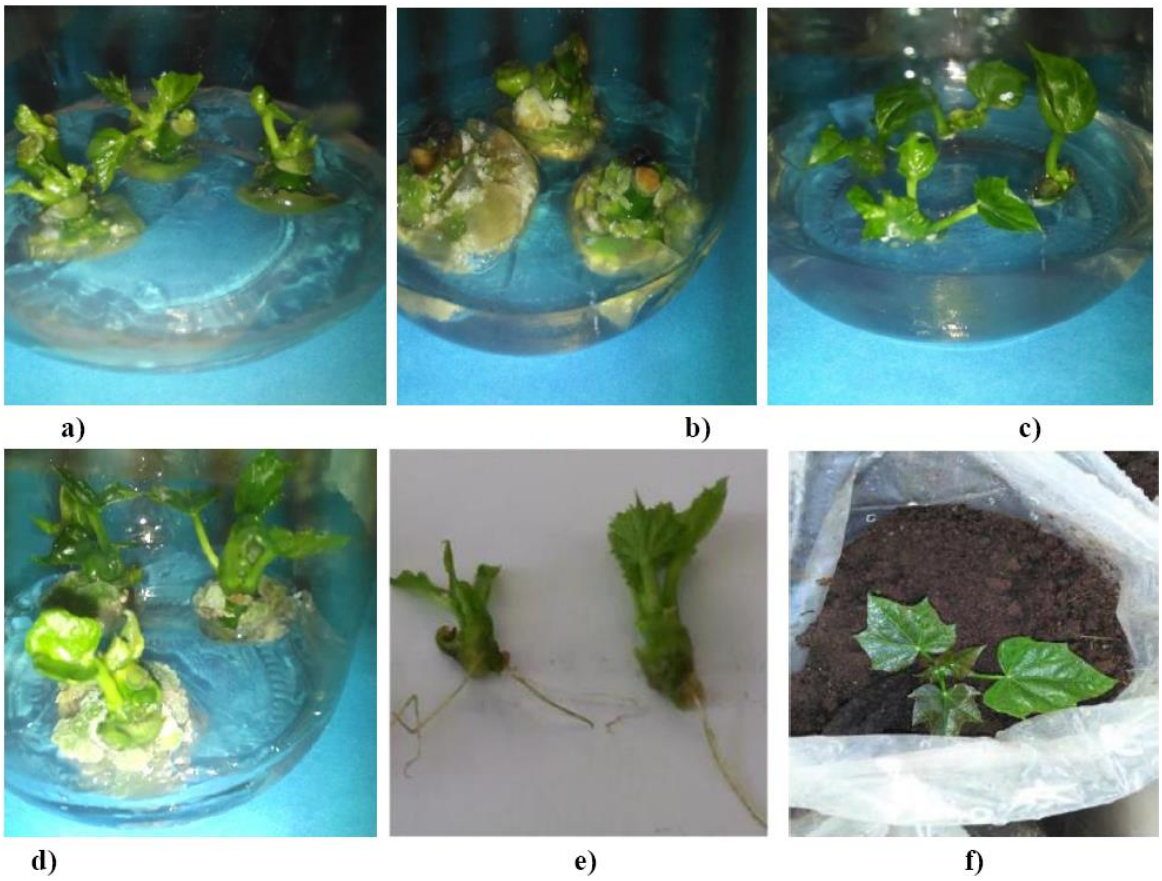

Figure 2. Plant regeneration in Jatropha through direct organogenesis. (a) Shoot induction on 1.5 mg/L of BAP from nodal explant, (b) MS medium containing 1 mg/L of IBA, (c) Shoot multiplication on 0.5 mg/L BAP, (d) Callus formation on rooting MS media with 0.5 mg/L NAA, (e) Rooting on half- strength medium with 0.25 mg/L IBA, (f) plantlet undergoing acclimatization.

combination of 1 mg/L of BAP and 1 mg/L IBA for all accessions (Table 2). Addition of IBA along with BAP has also been reported to regenerate shoot buds from the nodal explants in Jatropha (Shrivastava and Banerjee, 2008). This is mainly due to the reason that, the hormone balance is apparently more important than the absolute concentration of any one hormone since plant hormones do not function in isolation within the plant body, but, instead, function in relation to each other (Deore and Johnson, 2008). Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue (Jha et al., 2007; Shrivastava and Banerjee, 2008; Purkayastha et al., 2010).

However, in this study good results were also obtained on MS medium which contains only BAP (2 mg/L). This could be due to the endogenous auxin which balances with cytokinin releasing the shoot buds from apical dominance. Several studies have reported that BAP is the most suitable cytokinin for shoot induction and multiplication in Jatropha. Maharana et al. (2012) have also reported that MS medium fortified with 8.0 µ*M* BAP regenerated shoot buds (6.2 shoots) from nodal explants of Jatropha. On the other hand, *in vitro* growing shoots of Jatropha had revealed a tendency of callusing when cultured on a medium fortified with sole IBA, at a concentration of 1 mg/L (Figure 2b). Hence we can say that an optimum ratio of cytokinin to auxin is essential for proper shoot bud formation. The study by Xiansong (2010) indicated that the sole effect of BAP, IBA and their interactions had significant effects on plant regeneration and type of regeneration in sweet potato. This is supported by many researches implying that the interaction between auxin and cytokinin is important for the regulation and guiding developmental processes, such as the formation and maintenance of the meristem and formation of callus which are crucial mechanisms for the establishment explant (Kalimuthu et al., 2007).

Effects of BAP and kinetin on shoot multiplication

The result showed that number of leaves per shoot was highly significantly ($P<0.01$) affected due to the main

Table 3. Main effect of the different concentrations and combinations of Cytokinins (BAP and Kn) on number of leaves per shoot of Jatropha accessions.

PGR concentration (mg/L)		Number of leaf per shoot
BAP	Kinetin(Kn)	
0	0	3.43±0.08^{hi}
0	0.5	4.00±0.05^{g}
0	1.0	4.50±0.09^{f}
0.5	0	7.00±0.09^{a}
0.5	0.5	6.43±0.09^{b}
0.5	1.0	5.00±0.05^{e}
1.0	0	5.53±0.10^{d}
1.0	0.5	6.00±0.17^{c}
1.0	1.0	4.13±0.06^{g}
1.5	0	2.97±0.03^{j}
1.5	0.5	3.67±0.11^{h}
1.5	1.0	3.30±0.05^{i}
LSD (5%)		0.27
CV (%)		7.93

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15). CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples.

effect of PGRs concentrations and combinations, however, the effect of accessions and interaction of the two factors affected the number of leaves non-significantly (P>0.05). ANOVA also showed that genotype (Jatropha accessions), PGRs and their interactions had very high significant (P<0.01) effects on average shoot length and average shoots number (Table 3).

The maximum mean number of leaf (7 per shoot) was recorded on MS media supplemented with 0.5 mg/L of BAP followed by (6.43 per shoot) on media supplemented with combination of 0.5 mg/L of BAP and 0.5 mg/L of Kn. Whereas, the lowest mean number of leaf (2.97 per shoot) was recorded for shoots induced on MS media supplemented with 1.5 mg/L of BAP (Table 3). In this study a significant influence of the type of cytokinins and their concentrations on the number of leaves per shoot was observed. As the concentration of Kn increased the number of leaf per shoot also increased. However, the cultures growing on the media containing higher BAP produced the smallest number of leaves. Kozak and Salata (2011) reported that the lower concentration of BAP (0.5 mg/L) produced maximum number of leaves (16.8 leaves) than other concentration of Kinetin, 2iP and TDZ in *Rheum rhaponticum*. Similarly, Behera et al. (2014) obtained highest number of leaf per shoot (7.1) on MS medium supplemented with 1 mg/L BAP and 1 mg/L IAA in Jatropha. More recently Rady et al. (2016) also reported that MS supplemented with combination of 0.5 mg/l BA and 0.05 mg/L IBA gave the highest number (14.67) of leaves per proliferated shoot bud of Jatropha after one month of cultivation in the growth room.

The maximum number of shoots was recorded for Metema (6 shoots) on MS media supplemented with combination of 0.5 mg/L BAP and 0.5 mg/L Kn followed by 5.70 and 5.56 shoots for Shewa Robit and Adami Tulu accessions, respectively, on the same growth regulator combination and concentration as for Metema. Whereas, the lowest mean number (1.88-1.96) of shoots of the experiment was recorded for all the three accession shoots on growth regulator free MS media (Table 4). In this study, the optimum combination of BAP and Kn which was 0.5 mg/L BAP and 0.5 mg/L Kn showed better response on number of shoot per explants for all accession than the other treatments. This might be due to cytokinins participate in the regulation of cell division which leads to shoot bud formation from the explant. This is in line with the earlier studies where in media containing kinetin in conjunction with BAP induced higher frequency of shoot multiplication and greater number of shoots in some perennial plants (Figueiredo et al., 2001; Baskaran and Jayabalan, 2005). Weaker effect on axillary shoot regeneration for all the three accessions was observed in hormone free treatments. Thus results confirmed with Thepsamran et al. (2008) who reported that exogenous application of cytokinins has become obligatory for induction of multiple shoot in Jatropha.

In case of shoot length, the maximum shoot length was recorded for Shewa Robit (3.2 cm) on media supplemented with 0.5 of mg/L Kinetin. Whereas, the lowest mean shoot length (1.8-1.84 cm) was recorded for all the three accession shoots developed on media

Table 4. Effect of different concentration and combination of BAP and Kn on number of shoots and shoot length of three Jatropha accessions.

Jatropha Accessions	Cytokinin (mg/L)		No. of shoot per explant	Length of shoot (cm)
	BAP	Kn		
Metema	0	0	1.96 ±0.04^{r}	3.00±0.00 ^{b}
	0	0.5	2.10 ±0.03^{pqr}	3.04 ±0.02^{b}
	0	1.0	3.00 ±0.00^{lm}	2.90 ±0.00^{c}
	0.5	0	4.60 ±0.10^{de}	2.48 ±0.02^{i}
	0.5	0.5	6.00 ±0.31^{a}	2.80 ±0.03^{e}
	0.5	1.0	5.50 ±0.16^{b}	2.60 ±0.00^{f}
	1.0	0	4.10 ±0.10^{fg}	2.18 ±0.02^{kl}
	1.0	0.5	3.72 ±0.09^{hi}	2.34 ±0.02^{j}
	1.0	1.0	3.40 ±0.03^{jk}	2.06 ±0.02^{mn}
	1.5	0	3.50 ±0.00^{ijk}	1.80 ±0.00^{q}
	1.5	0.5	2.78 ±0.02^{mn}	2.10 ±0.03^{m}
	1.5	1.0	2.40 ±0.00^{op}	1.92 ±0.05^{p}
Adami Tulu	0	0	1.88 ±0.05^{r}	3.00 ±0.00^{b}
	0	0.5	2.06 ±0.06^{qr}	3.02 ±0.02^{b}
	0	1.0	2.84 ±0.10^{m}	2.87 ±0.02^{cd}
	0.5	0	4.00 ±0.00^{fgh}	2.50 ±0.00^{hi}
	0.5	0.5	5.56 ±0.23^{b}	2.82 ±0.02^{de}
	0.5	1.0	4.70 ±0.20^{cd}	2.56 ±0.02^{fgh}
	1.0	0	3.80 ±0.12^{ghi}	2.20 ±0.03^{k}
	1.0	0.5	3.68 ±0.07^{ij}	2.34 ±0.02^{j}
	1.0	1.0	3.33 ±0.00^{k}	2.10 ±0.00^{m}
	1.5	0	3.30 ±0.12^{kl}	1.80 ±0.00^{q}
	1.5	0.5	2.80 ±0.00^{mn}	2.06 ±0.02^{mn}
	1.5	1.0	2.34 ±0.04^{opq}	1.96 ±0.04^{op}
Shewa Robit	0	0	1.92 ±0.05^{r}	3.00 ±0.00^{b}
	0	0.5	2.02 ±0.08^{r}	3.20 ±0.00^{a}
	0	1.0	2.96 ±0.04^{m}	2.92 ±0.02^{c}
	0.5	0	4.30 ±0.12^{ef}	2.52 ±0.02^{ghi}
	0.5	0.5	5.70 ±0.12^{ab}	2.80 ±0.00^{e}
	0.5	1.0	5.00 ±0.00^{c}	2.58 ±0.02^{fg}
	1.0	0	4.00 ±0.16^{fgh}	2.22 ±0.02^{k}
	1.0	0.5	3.80 ±0.00^{ghi}	2.36 ±0.04^{j}
	1.0	1.0	3.53 ±0.12^{ijk}	2.12 ±0.02^{lm}
	1.5	0	3.70 ±0.12^{hij}	1.84 ±0.04^{q}
	1.5	0.5	2.84 ±0.04^{m}	2.10 ±0.00 ^{m}
	1.5	1.0	2.50 ±0.16^{no}	2.00 ±0.00^{no}
	CV (%)		6.98	**2.03**
	LSD (5%)		0.30	**0.06**

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5). CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error.

supplemented with 1.5 mg/L BAP (Table 4). On the other hand, the highest mean shoot length for Metema and Adami Tulu accession were obtained from both MS medium containing 0.5 mg/L Kn and PGRs free MS medium with no significant difference to the accessions type as well as hormone concentrations from which the shoot buds were raised. The increased response of shoot length to PGRs free media could partly relate to the

endogenous levels of hormone in explants. On the other hand, the impact of Kn on shoot length of all the three accession showed better response than the other treatments. This result also confirmed with Jeevan et al. (2013) who reported that 1.0 mg/L kinetin gave the highest shoot length than media which containing BAP alone during *in vitro* culture of Jatropha. This shows that Kn is effective in driving shoot elongation and this is might be due to the reason that Kn is easy and readily to be absorbed by plant cells as compared to BAP. Kaminek (1992) also reported that variation in the activity of different cytokinins can be explained by their different uptake rate in different genomes, translocation rates to meristematic regions and metabolic processes in which cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds.

Effects of IBA and NAA on rooting induction

ANOVA showed very highly significant ($P<0.01$) effect of all main and interaction effect of accession, IBA and NAA on rooting percentage and root length of *in vitro* rootinduction of the three Jatropha accessions. The result showed also that number of roots induced per shoot was highly significantly ($P<0.01$) affected due to the main effect of PGRs concentrations and combinations, however, the effect of accessions and interaction of the two factors affected the number of roots non-significantly ($P>0.05$).

The highest rooting percentage (88%) was recorded for Shewa Robit accession on media supplemented with 0.25 mg/L IBA followed by 86 and 84.8% for Metema and Adami Tulu accessions, respectively, on the same IBA concentration as for Shewa Robit. Whereas, the lowest rooting percentage (38.6-40.2%) were recorded for all accession shoots induced on media supplemented with combination of 1 mg/L IBA and 0.5 mg/L NAA (Table 5). On other hand, there is no root formed on the media supplemented with NAA alone. On this medium NAA often induces the formation of callus at the base of the shoot and rooting response was almost negligible (Figure 2d). Similar results to the present study were obtained by Shrivastava and Banerjee (2008) and Maharana et al. (2012). These authors observed that well-developed shoots of Jatropha when transferred to half MS medium fortified with NAA intermittent callus formation takes place and no roots were observed. The reason for low performance of NAA treatments may be due to the reason that NAA is more persistent than IBA, remains present in the tissue and may block further development of root meristemoids (Nanda et al., 2004). Studies also showed that, NAA was more consistent in stimulating cell divisions which favors callus formation (Kim et al., 2003). The promotory effect of IBA on *in vitro* rooting of shoots has also reported in Jatropha (Rajore and Batra, 2005; Kochhar et al., 2005) and they concluded that IBA alone was effective in the rooting of Jatropha.

The maximum mean root length was obtained for Shewa Robit (4.3 cm) on the media supplemented with 0.25 mg/L IBA followed by 4 and 3.8 cm for Metema and Adami Tulu accession, respectively on the same hormone concentration as for Shewa Robit. Meanwhile, the lowest root length (1.88-1.92) of the experiment was recorded for all the three accession shoots developed on hormone free half MS media (Table 5). In this study, the optimum level of IBA concentration is 0.25 mg/L for all the three accessions. Datta et al. (2007) have also reported that half MS medium fortified with 0.2 mg/L regenerated better root length (8.7 cm) from nodal explants of Jatropha. The results also revealed that root length tend to reduce with higher than optimum concentration of IBA. Kollmeier et al. (2000) reported that root elongation phase is very sensitive to auxin concentration and it is inhibited by high concentration of auxin in the rooting medium. It is possible that supra-optimal concentration of auxins inhibit root elongation through enhancement of ethylene biosynthesis which is root growth inhibitor (Hartman et al., 2009).

In case of root number the maximum root number (5.43) was recorded on MS media supplemented with 0.25 mg/L of IBA followed by (4.8) on media supplemented with combination of 0.5 mg/L of IBA and 0.25 mg/L of NAA (Table 6). Whereas, the lowest mean number (1.8) of roots per shoot was recorded for roots induced on PGR free MS media. On other hand, there is no root formed on the media supplemented with NAA alone due to callus formation at the base of shoot. In this study the root number decreased with higher concentration of IBA. These results are in line with Datta et al. (2007), who reported 0.2 mg/L IBA for best rooting in Jatropha. However, there are other studies who recommended 3 mg/l IBA addition to half MS medium for best rooting in Jatropha (Shrivastava and Banerjee, 2008). This deviation is owed the variation to the underlining genetic differences of the genotypes in response to rooting media composition and affecting rooting and other associated developments.

Besides, the decline in root number beyond the optimum level in this study might be due to the toxic effect of IBA beyond certain level which affects root growth and development. This observation is in agreement with the report of Thomas (2007) in *Curculigo orchioides* where a higher level of IBA produced a negative effect resulting in lower root number. Moreover, high levels of IBA can result in ethylene accumulation in the tissue culture vessel, which also inhibits the induction of root primordia (De Klerk, 2002; Hartman et al., 2009).

Acclimatization

The acclimatization results revealed that the highest percentage of survival rate of shoots (86.67%) was recorded for Shewa Robit plantlets whereas 73.33 and 66.67% recorded for Adami Tulu and Metema

Table 5. Effects of different concentration and combination of IBA and NAA on rooting percentage and root length of three Jatropha accessions.

Jatropha accession	Auxin (mg/L)		Rooting response (%)	Root length (cm)
	IBA	NAA		
Metema	0	0	51.20 ±0.66^{mn}	1.92 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	86.00 ±0.63^{b}	4.00 ±0.00^{b}
	0.25	0.25	76.200 ±0.20^{f}	2.74 ±0.10^{gh}
	0.25	0.5	64.00 ±0.55^{ij}	2.58 ±0.05^{hi}
	0.5	0	78.80 ±0.37^{e}	3.20 ±0.12^{e}
	0.5	0.25	84.20±0.20^{cd}	3.62 ±0.07^{cd}
	0.5	0.5	59.20 ±0.58^{k}	2.30 ±0.12^{jkl}
	1.0	0	71.60 ±0.51^{h}	3.00 ±0.00^{ef}
	1.0	0.25	54.40 ±0.68^{l}	2.26 ±0.06^{jklm}
	1.0	0.5	40.20 ±0.49^{o}	2.08 ±0.02^{lmn}
Adami Tulu	0	0	50.40 ±0.60^{n}	1.88 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	84.80 ±0.86^{bc}	3.80 ±0.00^{bc}
	0.25	0.25	74.80 ±0.73^{g}	2.87 ±0.03^{fg}
	0.25	0.5	63.60 ±0.24^{j}	2.48 ±0.13^{ij}
	0.5	0	80.00 ±0.00^{e}	3.50 ±0.16^{d}
	0.5	0.25	83.20 ±0.37^{d}	3.60 ±0.24^{cd}
	0.5	0.5	59.40 ±0.40^{k}	2.36 ±0.10^{ijk}
	1.0	0	72.00 ± 0.00^{h}	2.80 ±0.12^{fgh}
	1.0	0.25	55.00 ±0.45^{l}	2.22 ±0.09^{klm}
	1.0	0.5	38.60 ±0.81^{p}	2.06 ±0.02^{mn}
Shewa Robit	0	0	52.20 ±0.20^{m}	1.88 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	88.00 ±0.00^{a}	4.30 ±0.00^{a}
	0.25	0.25	76.00 ±0.32^{fg}	2.79 ±0.09^{fgh}
	0.25	0.5	65.00 ±0.45^{i}	2.74 ±0.02^{gh}
	0.5	0	76.60 ±0.98^{f}	3.60 ±0.10^{cd}
	0.5	0.25	83.60 ±0.68^{cd}	3.68 ±0.07^{cd}
	0.5	0.5	60.00 ±0.00^{k}	2.42 ±0.05^{ijk}
	1.0	0	71.60 ±0.51^{h}	3.10 ±0.10^{e}
	1.0	0.25	55.60 ±0.24^{l}	2.40 ±0.00^{ijk}
	1.0	0.5	40.00 ±0.55^{o}	2.04 ±0.02^{mn}
CV(%)			**1.89**	**7.63**
LSD (5%			**1.29**	**0.22**

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).CV = Coefficient of variation, LSD = Least Significant Difference, SD= Standard Error.

respectively after 30 days of acclimatization (Table 7). Loss of some plantlets might be due to differences in the genotype in adaptation to the new environment (*ex vitro*). The less development of cuticle under *in vitro* condition and the drop in relative humidity from near 100% in the culture vessels to much lower values in the greenhouse

Table 6. Number roots per shoots as affected by different concentration and combination of Auxins (IBA and NAA).

PGR concentration (mg/L)		Number of roots per shoot
IBA	NAA	
0	0	1.80±0.14[i]
0	0.25	-
0	0.5	-
0.25	0	5.43±0.08[a]
0.25	0.25	4.07±0.12[d]
0.25	0.5	3.31±0.06[e]
0.5	0	4.53±0.08[c]
0.5	0.25	4.80±0.11[b]
0.5	0.5	2.87±0.13[f]
1.0	0	3.50±0.05[e]
1.0	0.25	2.47±0.06[g]
1.0	0.5	2.07±0.12[h]
LSD (5%)		0.25
CV (%)		12.28

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15). LSD=Least Significant Difference, CV= Coefficient of variation, SD= Standard Error and n=number of samples, '-' indicates no response.

Table 7. Survival rate of plantlets derived from *in vitro* regeneration through direct organogenesis of three Jatropha accessions during acclimatization.

Accession	Total no. of plants acclimatized	No. of plants survived	No. of died plants	% of survived Plants	% of died plants
Adami Tulu	15	10	5	66.67	33.33
Metema	15	11	4	73.33	26.67
Shewa Robit	15	13	2	86.67	13.33

might result inexcessive water loss and death (Biradar et al., 2009). The current result is in agreement with the report of Jeevan et al. (2013) who declared 87% greenhouse acclimatization potential of *in vitro* generated Jatropha cultures. There were no observable variations with respect to morphological and growth characteristics between *ex vitro* sown parent plants and *in vitro* raised plants in pots.

Conclusion

This study provided optimal protocol for i*n vitro* mass propagation of Jatropha accessions viz. Metema, Adami Tulu and Shewa Robit accession through direct organogenesis from nodal explant. The present study concluded that best shoot induction from nodal explant was obtained from MS medium containing 1 mg/L BAP and 0.5 mg/L IBA for all the three accessions. MS media supplemented with combination of 0.5 mg/L BAP and 0.5 Kn mg/L followed by 0.5 mg/L BAP is sufficient for maximum shoot multiplication and growth parameters for the three accessions. On the other hand, the longest shoot length for Metema and Adami Tulu accession were obtained from both MS medium containing 0.5 mg/L Kinetin and PGRs free MS medium with no significant difference. That means free MS media can also be used for shoot elongation. Among various concentration and combination of auxin tested, half MS medium supplemented with 0.25 mg/L IBA followed by 0.5 mg/L IBA with 0.25 mg/L NAA were best for all root induction and growth parameters for the three accessions. *In vitro* induced shoots were well rooted and successfully established in green house environment. Generally, in this study an efficient direct organogenesis protocol was developed for Jatropha and this technique can be used

as guidelines for improving *in vitro* mass propagation of the crop.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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