In Vitro Regeneration Protocol through Direct and Indirect Organogenesis for Jatropha (*Jatropha curcas*) Accessions in Ethiopia

MSc. THESIS

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COLLEGE OF AGRICULTURE

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This is to certify that the thesis entitled **"*In Vitro* Regeneration Protocol through Direct and Indirect Organogenesis for Jatropha (*Jatropha curcas*) Accessions in Ethiopia"** submitted in partial fulfillment of the requirements for the degree of **Master's** with specialization in **Horticulture,** the Graduate Program of the **School of Plant and Horticultural Science, Hawassa University College of Agriculture** and has been carried out by **Hundessa Fufa** under my/our supervision. Therefore I/we recommend that the student has fulfilled the requirements and hence hereby can submit the thesis to the school.

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DEDICATION

I dedicate this thesis to my mother and father. Without their support I would not have reached this stage.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my original work and all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirement for M.Sc. degree at Hawassa University and is deposited at the University Library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that an accurate acknowledgement of the source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the Head of the school of Plant and Horticultural Sciences or the Dean of the School of Graduate Studies when in his or her judgment the proposed use of material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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LIST OF ABBREVIATIONS AND ACRONYMS

2, 4-D	2, 4-dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6-Benzyl-amino-purine
CRD	Completely Randomized Design
FDRE	Federal Democratic Republic of Ethiopia
GA_3	Gibberellic acid
HCl	Hydrochloric Acid
IBA	Indole-3-butyric acid
Kn	Kinetin
LSD	Least Significant Difference
mg/l	milligram per litre
MME	Ministry of Mine and Energy
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
NABRC	National Agricultural Biotechnology Research Center
NaOH	Sodium Hydroxide
PGRs	Plant Growth Regulators
pH	Power of Hydrogen
RH	Relative Humidity
SARC	Sirinka Agricultural Research Center
SAS	Statistical Analysis System
TDZ	Thidiazuron
V/V	Volume per volume
W/V	Weight per volume
WGRC	Wondo Genet Research Center

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In Vitro Regeneration Protocol through Direct and Indirect Organogenesis for Jatropha (_Jatropha curcas_) Accessions in Ethiopia

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ABSTRACT

Jatropha curcas L. is among the important tree crops in the world with a potential for biofuel production. The crop is drought resistant and thrives well in warm tropical climates. Ethiopia has favorable environment for Jatropha production and there is a soaring investors' interest to produce Jatropha in the country for biodiesel production. However, insufficient good quality of propagation material is a major production constraint. In line with this, a study was undertaken to establish a protocol for in vitro mass propagation of Ethiopian Jatropha using three accessions, viz. Metema, Adami Tulu and Shewa Robit accession through direct and indirect organogenesis. The experiment was laid out in CRD with five replications in factorial arrangement. Nodal and leaf explants were used as explants for direct and indirect organogenesis, respectively. Local bleach (Berekina) at a concentration of 2.5% and 3% for 15min found to be effective for sterilization of leaf and nodal explants, respectively. For direct organogenesis, the highest percentage of shoot induction (86-90%) was achieved when on MS medium with BAP (1.5mg/l) and IBA (0.5 mg/l) for all the three accessions. The maximum (6) number of shoots was obtained for Metema when BAP (0.5mg/l) with Kn (0.5mg/l) was used. Whereas, the maximum (3.2cm) shoot length was recorded for Shewa Robit on media with 0.5mg/l Kn. The highest rooting percentage (84.8-88%) for all accessions and maximum root number (5.43) were recorded on media supplemented with 0.25mg/l IBA. The maximum root length was observed for both Shewa Robit (4.3cm) and Metema (4cm) on media with 0.25mg/l IBA. Whereas, maximum root length (3.8cm) was achieved on media with combination of 0.5mg/l IBA and 0.25mg/l NAA for Adami Tulu accession. For indirect organogenesis, in vitro regeneration protocol was partially achieved through regeneration of adventitious shoots from leaf-derived callus tissue. The medium supplemented with combination of 1mg/l BAP and 1mg/l 2,4-D resulted in maximum percentage of callus (100%) formed for all accessions. The maximum shoot regeneration (66.67%) from callus with 10.13 number of shoot was obtained from Shewa Robit in MS medium fortified with TDZ (0.5 mg/l) and IBA (0.1mg/l). In shoot multiplication, the maximum shoot number (3.5) was obtained from Shewa Robit on media with 0.5mg/l BAP. Whereas, the maximum shoot length was recorded (2.1-2.26cm) for all accessions on media supplemented with combination 0.5 of mg/l BAP and 0.5mg/l Kn. However, the elongated shootlet which transferred into half MS medium containing various concentrations of IBA and NAA failed to induce root growth. On the other hand, micro shoots regenerated via direct organogenesis were well rooted and successfully established in green house environment 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. In order to see further achievement in vitro propagation of Jatropha, it is imperative to include additional accessions and combinations of PGRs.

Key Words: *Jatropha curcas, Biofuel, Callus, Organogenesis, Plant Growth Regulators*

1. 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).

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 (*Jatropha 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; FDRE, 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 were 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 that 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 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 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 developing of an efficient *in vitro* regeneration system would be a remarkable progress for the Jatropha business and the field of alternative energy technology (Rajore *et al.,* 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, 1990; Hussain *et al.*, 2012; Moniruzzaman *et al.*, 2016). The

unipolar structure is either a shoot or a root and is physically connected to the tissue of origin. 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, 1990; Geetha *et al.*, 2008). Direct organogenesis involves the emergence of adventitious organs directly from the meristematic region of the explants without callus phase. In contrasts, adventitious organs are produced through a callus phase in indirect organogenesis. Organogenesis process is much common than somatic embryogenesis and has far more potential for mass clonal propagation of plants (Shen, 2007). Direct organogenesis is important to ensure clonal fidelity whereas; indirect organogenesis often shows genetic variation which is useful to regenerate new plants from somaclonal variants obtained through callus and also initiate propagation from non-meristematic tissues (Sridhar and Naidu, 2011; Jose *et al.*, 2012). 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). Both callus induction and plant regeneration from explants require 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 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 and indirect organogenesis of three Ethiopian Jatropha accessions using nodal and leaf explants.

Hence, the specific objectives of the study were;

- To standardize the concentration and duration of exposure of local bleach (Berekina) for surface sterilization of leaf and nodal explants;
- To optimize the concentrations and combinations of different growth regulators on MS medium for maximum proliferation of shoots from both direct and indirect organogenesis;
- To determine the optimal concentrations and combinations of plant growth regulators for callus induction;
- To investigate rooting response of shoots to different IBA and NAA concentrations and combinations;
- To evaluate survival rates of acclimatized plant regenerants in green house environment.

2. LITERATURE REVIEW

2.1. Origin, Distribution and Taxonomy of Jatropha

A number of researchers have attempted to define the origin of Jatropha, but the source remains controversial. Martin and Mayeux (1984) proposed Brazil as center of origin but without giving any arguments. However, Aponte (1978) indicated Mexico and Central America as center of origin to physic nut. Jatropha was spread by Portuguese traders as a valuable hedge plant via the Cape Verde Island and Guinea Bissau (Heller, 1996). Then it distributed to different countries like Africa, Asia, and India (Jepsen *et al.*, 2006).

Jatropha curcas is commonly called physic nut, purging nut, barbados nut, pig nut or fig nut (Benge, 2006). Taxonomists classified *Jatropha* as the genus but most literature simply refer to the plant as Jatropha, adding it to the list of common names. According to Dehgan and Webster (1979), the taxonomic hierarchy of Jatropha is mentioned below.

Kingdom	*Plantae*
Phylum	*Magnoliophata*
Class	*Magnoliopsida*
Order	*Malpighiales*
Family	*Euphorbiaceae*
Genus	*Jatropha*
Species	*Curcas*

Jatropha is a bush or small tree and belongs to the Euphorbia family. The genus *Jatropha* contains approximately 170 known species. The genus name *Jatropha* derives from the Greek ***jatros*** (doctor), ***trophe*** (food), which implies medicinal uses. It has chromosome numbers of 2n = 2x = 22 and a basic chromosome number of x = 11(Puangpaka and Thaya, 2003). It is a small tree or a large shrub which can achieve a height of 5 meter and rarely can attain a height of 10 meter under favorable conditions.

2.2. Use of Jatropha

Jatropha is a drought resistant multipurpose perennial plant. Commercially, it has increased its demand beyond imagination. The plant consists of the stem (woody part), the leave, the root and other reproductive organs that have one or more use. The seed of Jatropha is the most useful part of the plant. Jatropha is actually a green gold in a shrub. It has numerous benefits which include:

2.2.1. Energy Source

Different parts of Jatropha can be used as source of energy. Common energy forms that can be acquired from different part of Jatropha include, biomass energy (charcoal), biogas (hydrocarbons of fruit and seed shells) and biodiesel (oil and methyl ester of the oil). Of all these fuel energy generated from Jatropha, energy from the seed is paramount and has attracted global attention. It was extensively studied that seed oil from the plant is used as an alternative stationary engine or transportation fuel (Achten *et al.,* 2008). This is due to its potential to substitute fossil diesel. The calorific value of seed oil is 39MJ kg-1 which is higher than anthracite coal and comparable to crude oil (Sotolongo *et al.*, 2009). Raw oil has been used as a substitute for petro-diesel both in modified and unmodified diesel engines.

2.2.2. Other Uses

a) Living Fence (Hedge)

Jatropha is an excellent hedging plant generally grown in most part of world as live fence for protection of agricultural fields against damage by livestock as unpalatable to most domestic animals. It also serves as wind break both at homestead and agricultural field. In general, the use of Jatropha as biofence has been reported as cost effective and lifelong compared to wire fence (Kumar and Sharma, 2008).

b) Green Manure and Fertilizer

Cultivation of Jatropha resulted in 11% average increase in mean weight diameter of the soil and 2% increase in soil macro-aggregate turnover (Chaudhary *et al.,* 2008) and regression analysis showed a significant correlation between organic carbon and mean weight diameter. Such soil structure recovery under cultivation of Jatropha implies a sustainable improvement

in the surface integrity of these soils, which will ensure more water infiltration rather than runoff and erosion. Such improvement of soil structure also increase activity of microorganisms that improve soil synthesis. The Jatropha seed cake which is a byproduct of oil extraction can be used as potential organic manure since it has nitrogen content ranging from 3.2-3.8 % (Kumar and Sharma, 2008).

c) Soap Making

Jatropha oil has a very high saponification value and is ideal for soap manufacture. The glycerin is a by-product of biodiesel which can be used to make soap, and also soap can be produced from Jatropha oil itself. In either case the process produces a soft, durable soap and is a simple one, well adapted to household or small-scale industrial activity (Openshaw, 2000).

d) As Food

Jatropha can be toxic when consumed due to the seeds contain toxic Phorbol esters. However, a non-toxic variety of Jatropha is reported to exist in some provenances of Mexico and Central America (Makkar *et al.*, 1998). This variety is used for human consumption after roasting the seeds/nuts. Nowadays, this non-toxic variety of Jatropha could be a potential source of oil for human consumption, and the seed cake can be a good protein source for humans as well as for livestock (Makkar *et al.*, 2009).

e) Medicinal Use

Jatropha is widespread in arid and semi-arid tropical region of the world and has been used as traditional folk medicine and for veterinary purpose in many countries. It is a source of several secondary metabolites of medicinal significance. The leaf, fruits, latex and bark contain glycosides, tannins, phytosterols, flavonoids and steroidal sapogenins that exhibit wide ranging medicinal properties that make pharmaceutically such as anti-bacterial and antifungal activities (Debnath and Bisen, 2008). And also all parts of the Jatropha plant show insecticidal properties against various insects like cotton bollworm, and for pests of pulses, potato and corn (Kaushik and Kumar, 2004).

2.3. Bio-safety of Jatropha as Biofuel Production

Increasing industrialization and motorization in both developed and developing countries are leading to spiraling increase in the demand of fossil fuel (Pimentel *et al.,* 2008). Over 80% of the total combusted energy is still coming from fossil fuel. Global oil production may likely reach a maximum between 2015 and 2030. It is then expected to sharply decline to 40% of today's production by 2075 (Demirbas and Demirbas, 2010). Beside the finite nature of fossil fuel reserves, the world is also anguishing from its emission related problems such as global warming, ozone layer depletion, air pollution, acid rain and the consequence of climate change: drought and flooding (Balat and Balat, 2009).

In addition to this, current biofuel production mainly uses the first generation feedstock as these materials are readily available. Corn, soybean, wheat, oil palm, rapeseed and sugar cane are the major biofuel feedstock currently used in biofuel production. These feedstocks are used widely for human consumption shares the same resources to produce food crops. This become a challenge to global food security and particularly, to those countries which are food insecure (Knothe, 2000). On the other hand, productions of these feedstocks require fertile land and intensive agricultural inputs. This also competes with a reserve agricultural land and exerts pressure on forest and woodland resources. Consequently, this causes land use change which results in greenhouse gas emission more than fossil fuel (Pimentel *et al.,* 2008). Due to this developing a reliable source of renewable energy is attracting a major global attention.

Use of non-edible feed stocks (such as Jatropha oil) production of second generation biofuels can solve limitations of first generation biofuel. Jatropha can grow on degraded land and gives desirable yield. The plant is resistant to many biotic (pest, disease) and abiotic (drought, high temperature, nutrient deficiency and salinity) environmental factors (Edrisi *et al.,* 2015). The fungal association of the plant root, high response of the plant to its seed cake and high soil nutrient turnover from defoliated leaves during dry season makes the plant best candidate of biofuel feedstock on degraded lands (Chaudhary *et al.,* 2008; Kumar and Sharma, 2008). Thus, if properly designed production of biofuel from Jatropha is ecological, socially and economically feasible.

2.4. Overview of Jatropha as Biodiesel Crop in Ethiopia

Jatropha grows in various parts of Ethiopia such as in Wolayita, Metekel, Southern Wollo, Northern and Eastern Shoa, Tigrai, Gamo Gofa zones and Gambella region (Getinet *et al*., 2009). It grows mainly in dry area and also it grows in mid and high altitude. Its local Amharic name is "*Gullo*", but farmers also refer to it as "*Ayderkie*", "*Yedinber Shimagilie*" and "*Yedinber Astaraki*", meaning "drought resistant", "border mediator" and "good farmland demarcation", respectively, and indicating farmers' primary uses for the plant (FDRE,2007; Zufan,2010). Traditionally, farmers in Ethiopia mainly used Jatropha plants as living fences and as a structural means of conserving soil and water. And also it is used as fuel for lamps, as fertilizer and for medicinal purposes in parts of northeastern Ethiopia (Zufan, 2010).

Now, in connection with green economy goals, the Ethiopian government has begun promoting cultivation of Jatropha to make biodiesel for transportation (FDRE, 2007; FDRE, 2011). "The Ethiopian Biofuel Development and Utilization Strategy" is targeted to supply fuels from locally produced biofuel and the objective of the strategy is to ensure the production of biofuel without affecting food self-sufficiency, to improve balance of payment and to reduce environmental impacts. The preferred feedstocks for producing biodiesel in Ethiopia are Jatropha, castor and palm oil, while ethanol is primarily produced from sugarcane (FDRE, 2007).

Based on this strategy local and foreign private investors have started growing plants for producing biodiesel. There are about 85 companies which were licensed in Ethiopia to invest in biofuels, mainly Jatropha (Mengistu, 2013).The strategy also stipulates that with the participation of 1.25 million farmers in Jatropha biodiesel production the imported 1 billion liters of petrol diesel per annum can be substituted. Recently, the Ethiopian Ministry of Water and Energy declared that a total of one million hectares of land were suitable for Jatropha cultivation. The country has designed a five-year Jatropha development project aimed at transplanting 700 million Jatropha seedlings and produce 500 million liters biodiesel (ENA, 2014). By involving biodiesel producing companies the production will be in excess of local consumption and it will be a good commodity for export (MME, 2008). In general, the Jatropha has received a glorious reception as a miracle plant. Huge plantation program is being launched in regional states of the country on the onset of the biofuel policy and strategy.

2.5. Production Constraints of Jatropha

2.5.1. Jatropha Agronomic Constraints

Jatropha is believed as a low input crop because of its ability to grow on barren land. However, it needs adequate nutrients as fertilizer and rainfall or irrigation for growing as a productive crop. Moisture and nutrients have larger influence on the seed yield and oil productivity from the plantation on marginal lands. The plant growth and the seed yield of Jatropha were significantly increased under irrigated conditions as compared to non-irrigated conditions (Tikkoo *et al.*, 2013). Application of nitrogen and phosphorus increased the growth, seed yield and oil yield of Jatropha (Patolia *et al.*, 2007). There is also inadequate information with respect to the planting patterns, plant spacing, intercropping, insects and diseases control and effects of management practices in Jatropha (Yu, 2007).

Comprehensive information on local pests and diseases attacking the plant is not yet available. There has been a long-standing belief that Jatropha is largely resistant to pests and diseases because of its toxicity, particularly in regions where it has been introduced, because local pests and diseases have not had the opportunity to co-evolve with the plant (Jongschaap *et al*., 2013). The argument fails to consider the multitude of pests and diseases, either generalist that have evolved to cope with a range of toxic plant compounds, or pests of closely-related species with similar defense mechanisms. There is now increasing evidence that Jatropha is highly susceptible to pests and diseases and that these may seriously hamper plant growth and seed production (Anitha and Varaprasad, 2012). Recent studies reported that the Jatropha plant was susceptible to viral infection (Cucumber mosaic virus), insect attack, rodents, powdery mildew, leaf spots, insect defoliations and fungal diseases of the soil (Everson *et al.*, 2013). These infections caused approximately 60‑80% damage to the standing Jatropha crop at different study sites (Terren, 2012).

2.5.2. Lack of Improved Cultivars

Among the many Jatropha production constraints, the most important include lack of improved cultivars and a poor seed supply system. There is high fluctuations of the unit seed yield and can result in oil concentration variations between 8–54% (Ovando-Medina *et al*., 2011). Therefore developing of a higher yielding and more oil containing variety is one of the

main effective solutions. However, a good commercial variety is still missing (Moniruzzaman *et al.*, 2016). Zhang *et al*. (2009) and Yu (2007) also point out that variety breeding is one of the main hurdles for Jatropha planting.

Actually, the current Jatropha breeding program is limited to conventional breeding and surveying of germplasm resources of wild Jatropha plants (Zhang *et al*., 2009). However, the study of modern biotechnology application on Jatropha improvement is limited (Moniruzzaman *et al.*, 2016). Particularly, studies on cloning, expression and biological function annotation for Jatropha genes, which are responsible for economical traits, are largely absent.

2.5.3. Propagation Method of Jatropha

Conventionally Jatropha is propagated through seed and vegetative cutting (Heller, 1996). Jatropha seeds are genetically heterozygous as Jatropha species forms artificial and natural hybrid complexes readily and poses a problem to genetic fidelity (Sujatha and Prabakaran, 2003). Propagation through seed (sexual propagation) leads to genetic variability in terms of growth, biomass, seed yield, and oil content. Jatropha seeds are oily and cannot be stored for long- seeds older than l5 months show viability below 50% (Kobilke, l989). Many factors influence the establishment by direct seeding of Jatropha such as, sowing time, depth of sowing, age and quality of seed, soil moisture content, and quality of soil preparation (Heller, 1996; Shrivastava and Banerjee, 2008). Due to its perennial nature, seed setting requires 2 to 3 years. Seed production ranges from about 0.3 to 10.9 tons per hectare per year (Openshaw, 2000).

Vegetative propagation has been achieved by stem cutting, grafting, and budding as well as by air layering techniques. The optimal stem for cutting is 0.08 inches (2.0 mm) in diameter and 30.4 cm in length. Distal portion of the stock plants exhibit reduced rooting potential, while cuttings from the lower or juvenile regions of the plants generally maintain a higher rooting capacity than those from the upper regions (Hartmann *et al*., 1990). The stem pieces can be cut from the mother plant and planted at any time of the year. If it is planted during a dry period, irrigation is required. Direct planting of Jatropha by cutting decreased the time of production as compared to direct seeding or transplanting but showed a lower longevity and a lower

drought and disease resistance than those propagated by seeds (Heller, 1996). However Jatropha produced from cuttings do not produce true taproots, rather, they produce pseudo taproots that may penetrate only one half to two thirds the depth of the soil compared to taproots produced on seed grown plants and hence are less drought tolerant (Heller, 1996). Therefore, propagation using cuttings is not the preferred method of most growers.

2.6. Plant Tissue Culture and Its Application in Jatropha

Jatropha is considered to be a better candidate for the production of biofuel because of its high oil content and has lately attracted particular attention as a tropical energy plant. Because of these physiological factors of possessing high oil content, it is important to improve its biomass production. For this reason, biotechnological techniques such as tissue culture should be utilized (Ali *et al*., 2015).

Plant tissue culture is a science of growing and maintaining of plant cells or organs in nutritionally and environmentally supportive conditions. *In vitro* techniques have been used for propagation of many plant species and to generate material for germplasm preservation, and crop improvement to increase field productivity and profitability (Thepsamran *et al*., 2006). Micropropagation is suitable for rapid and large-scale clonal multiplication of elite germplasm (Mohan and Haggman, 2007). The technique has been referred to as micropropagation because the size of the tissue in culture is very small as compared to conventional vegetative cutting or any other plant part (Hartmann *et al*., 1990).

The scope of micropropagation was further extended to vast range of plant species, including fruit and plantation crops and forest species trees. Through micropropagation, the disadvantage of *in vivo* vegetative propagation such as, transmission of diseases is possibly avoided. Moreover, micropropagated plants exhibit vigorous growth and higher yields, and help in reducing the breeding cycle. Under *in vitro* conditions, production of homozygous plant is possible (George, 2008).

The application of plant tissue culture is highly beneficial in plants in which vegetative propagation is not possible or the propagation rate is very slow (Aldriance and Brison, 2000). Tissue culture of Jatropha was undertaken to circumvent the problems associated with large scale multiplication (Sujatha and Mukta, 1996). Jatropha species are amenable to tissue culture

manipulations, which indicate scope for widening the genetic base through parasexual hybridization and biotechnological tools (Sujatha, 2006). Evaluations of tissue culture propagated plants of Jatropha indicate that they have an advantage over seed propagated plants in terms of yield and yield related traits (Sujatha *et al*., 2005). Farmers who adopt *in vitro* propagated material may benefit from increased income through reduced pest control costs and higher effective yields (Muyanga, 2009). In general, clonal propagation by tissue culture is important for production of genetically superior planting material.

2.7. Plant regeneration Studies via Organogenesis in Jatropha

Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissue, which is not easily separated unless cut-off (Geetha *et al*., 2008).

There are two types of shoot organogenesis, direct and indirect. Direct shoot organogenesis is the production of shoots directly from the meristematic primordia of the explant without an intervening proliferative callus stage, while indirect refers to the formation of shoots indirectly from an intermediary callus that first develops on the explants (Kane *et al*., 1994).

The major distinction between the two pathways is, of course, the presence or absence of a discernible callus stage early in the morphogenic sequence of events. Callus is actively dividing non-organized masses of undifferentiated and differentiated cells often developed in the presence of growth regulators (Kumar *et al.*, 2015). The callus tissue, containing cells that have dedifferentiated into a less determined, morphologically more flexible form, serves as the starting point for *de novo* organogenesis. In the absence of a callus intermediate stage, cells present within the explant have the capacity to act as the direct precursors of the new primordium (Moniruzzaman *et al.*, 2016). However, both of the pathways have their own advantages. Direct organogenesis allows for faster and more successful regeneration of plants *in vitro* (Hicks, 1980; Sridhar and Naidu, 2011). Axillary bud proliferation is one of the most types of direct organogenesis and it is the techniques most widely used in commercial micropropagation and which show the least variation among the propagated plants (Gamborg and Phillips, 1995). Indirect organogenesis provides a means of dedifferentiating plant tissue

into callus, allowing for transformation and selection protocols, and regenerating whole plants *in vitro* from transformed calli with higher propagation rates (Robinson and Firoozabady, 1993; Jose *et al.*, 2012).

Shoot organogenesis offers several advantages compared to somatic embryogenesis viz; shoot organogenesis is more easily achieved in most species than somatic embryogenesis; shoot production is more synchronous than somatic embryo formation; indirect shoot organogenesis is associated with higher genetic variability and has more chance for selection of somaclonal variants; survival rate is higher among plantlets produced via shoot organogenesis than those via somatic embryogenesis after transferring to greenhouse (Shen, 2007).

For *in vitro* regeneration system of Jatropha, both direct and indirect methods have been reported using different explants such as cotyledon, petiole, hypocotyl, epicotyl, leaf tissue (Sujatha and Mukta, 1996; Wei *et al.*, 2004; Zhong Guang *et al.*, 2012; Singh *et al.*, 2014), axillary buds (Sujatha *et al.*, 2005), shoots tips (Rajore and Batra, 2005), nodal explant (Kalimuthu *et al.*, 2007; Maharana *et al.*, 2012) and Cotyledonary petiole (Liu *et al.*, 2016).

2.7.1. Developmental Pathways of *In vitro* Organogenesis in Plants

The process of organogenesis involves three main phases: dedifferentiation, induction, and re-differentiation (Shen, 2007). De-differentiation is the reversion of a specialized cell or tissue to an unspecialized form. In the direct pattern, specific cells directly become competent for organ formation without a callus phase. In the indirect pattern, there is an intervening cell division (callus) stage in which these new cells become competent. Competent cells are then able to respond to an induction treatment and progress toward the attainment of an organ specific fate. Once this organ specific fate is acquired, the cells are considered determined to move into re-differentiation phase of organogenesis and produce new organ (Thorpe, 1990).

The span between the time cells become competent and when they become fully determined for primordial production refers to as the induction phase (Schwarz and Beaty, 2000). During the induction phase, a competent cell or a group of competent cells become committed to a unique developmental fate on the stimulus of inducing signal. At the end of induction phase, cells become fully determined and capable of shoot organogenesis even with the removal of inducing signals (Schwarz and Beaty, 2000). Re-differentiation is the process by which a

group of once differentiated cells return to their original specialized form and function with the ability to form a whole plant (Geetha *et al*., 2008). During this phase, morphological differentiation and development of the nascent organ occur.

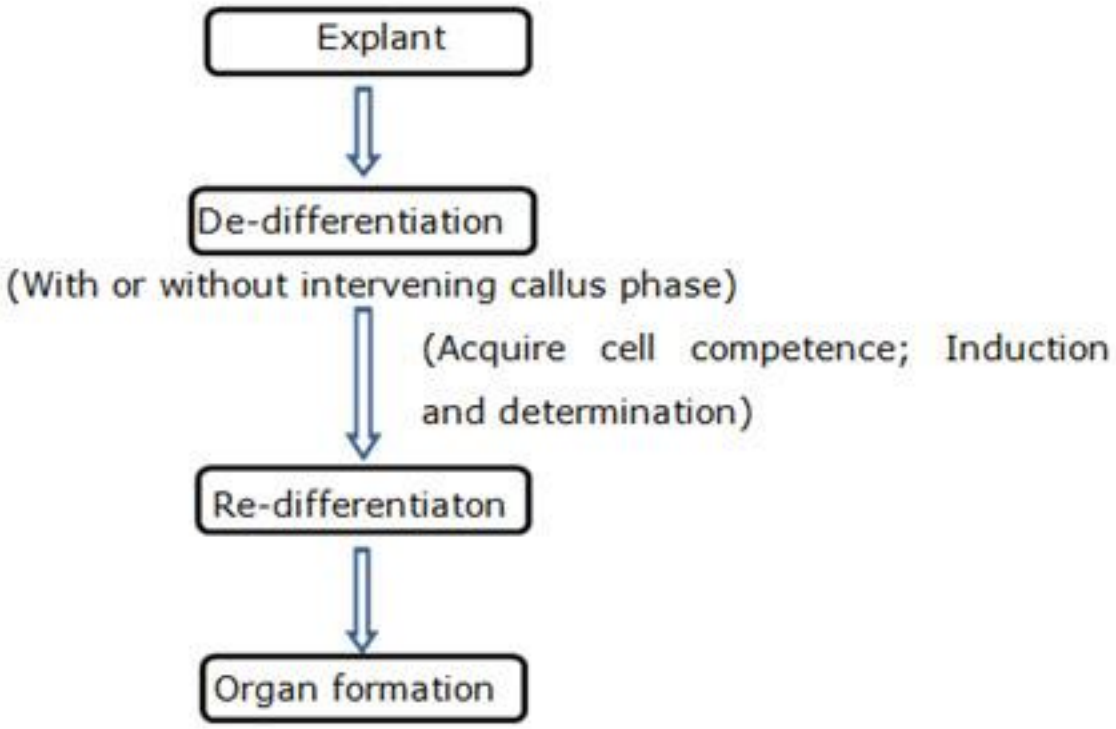

Figure 1. A scheme to describe the events resulting in adventitious organogenesis (Shen, 2007).

2.7.2. Factors Affecting Organogenesis of Jatropha

There are many factors which affect explant tissues becoming competent, and eventually determined to complete shoot formation. Those factors are such as; growth media, PGRs, nutrient and vitamins, energy source, source and type of explants, genotype, and culture condition, etc. (Deore and Johnson, 2008). However, by considering the objective of the study the following factors were briefly mentioned.

2.7.2.1. Genotype of the Source Plant

Genotype is one of the most important factors affecting plant regeneration *in vitro* (Tyagi *et al*., 2001; Feyissa *et al*., 2005; Landi and Mezzeti, 2006). Different genotypes respond

differently to regeneration even when subjecting the same culture environment. Some genotypes are easily cultivated via shoot organogenesis while others are either recalcitrant or exhibit no capacity for shoot organogenesis (Landi and Mezzetti, 2006).

Several researchers reported that *in vitro* regeneration in Jatropha is highly genotype dependent (Kumar, 2008; Sharma *et al*., 2011; Kumar and Reddy, 2012; Mweu *et al*., 2016). Sharma *et al*. (2011) reported that out of four toxic and one non-toxic Jatropha genotypes studied, non-toxic was least responsive (54.9%) with small number of shoots (4.0) per explants. Kumar and Reddy (2012) also reported that significant differences in the percentage of induction of shoot buds and the number of induced shoot buds per explant was observed among three Jatropha genotypes studied. Among the three genotypes, best shoot buds induction (66.97%) and number of shoot buds (13.76) per explant was observed from CSMCRI-JC-3 genotype of Jatropha. More recently Mweu *et al*. (2016) reported that the response of growth hormones in the culture media was variable within genotype and explant in five Jatropha accessions.

Generally, this differential behavior can be related to different mechanisms for control of the endogenous PGRs metabolism and/or contents. Cells within the same plant can have different endogenous levels of plant growth regulators and variation in receptor affinity (Schween and Schwenkel, 2003), thus it is reasonable to expect that *in vitro* response will vary with genotype. And also Phillips (2004) concluded that specific genes were involved in dedifferentiation, acquisition of competence and induction stages of shoot organogenesis. Some genotypes may lack the genes required for shoot organogenesis or these genes may not be functional.

2.7.2.2. Type of Explant

The correct choice of explant material can have an important effect on the success of tissue culture. The developmental stage and type of explants play a critical role in the success of organogenesis (Hartmann *et al.,* 1990).Type of explant is one of the important factors in optimizing the tissue culture protocol. Type of explants like leaf, petiole, cotyledonary leaf, hypocotyl, epicotyl, embryo, node and root explant significantly effect on tissue culture process and regeneration type of the plants (Sujatha and Mukta, 1996; Tyagi *et al*., 2001;

Kumar *et al*., 2011). This may be due to the different level of endogenous plant hormones present in the plants parts and the developmental regeneration the explants would follow depending on from which plant part they were excised. Leaf is the most commonly used explant for regeneration due to more surface area available to evoke regeneration (Sujatha and Muktha, 1996; Tyagi *et al*., 2001).

Several authors in different experimental set up and in various species has shown that maximum regeneration efficiency was observed from leaf explants that when taken other tissues and organs such as roots, shoots, hypocotyl, petiole and internodes (Sujatha and Mukta, 1996; Tyagi *et al*., 2001; Kumar *et al*., 2010a). Tyagi *et al*. (2001) have shown that using leaf as explants increase the regeneration rate and processed as compared to explanted derived from root, shoot, and leaf explant of *Cajanus cajan* plant. Also in Jatropha *in vitro* regeneration experiment used different explant like leaf, petiole and hypocotyl, the maximum regeneration frequency was observed from leaf explants (Sujatha and Mukta, 1996; Kumar *et al*., 2010a).

In addition to plant regeneration from leaves, nodal culture is also one of the most effective and common for *in vitro* methods in micropropagation of Jatropha (Datta *et al*.,2007; Shrivastava and Banerjee, 2008; Jeevan *et al*., 2013). Maharana *et al.* (2012) have also reported that highest shoot buds number (6.2) was obtained from nodal explants of Jatropha. It comprises a tissue culture approach, which entails culturing of nodal segments carrying axillary buds and stimulating them to develop and proliferate *in vitro*. Since this method involves the exploitation of buds already present on the parent stock plant, it provides an efficient means of clonal multiplication and plants raised from these are comparatively more resistant to genetic variation (Datta *et al*., 2007).

2.7.2.3. Plant Growth Regulators

Plant growth regulators (PGRs) are the most important in inducing signal for shoot organogenesis. They play a crucial role in determining the pathway of the plant cells. Dedifferentiation, induction and development of shoots or roots are regulated by both endogenous and exogenous growth regulators. The type of PGRs and their interaction play an important role in dedifferentiation, induction and development of shoots or roots (Khanam *et*

al., 2000). It is generally believed that cytokinins are beneficial for shoot formation, while auxins stimulate callus formation, root formation and somatic embryogenesis. The ratio of cytokinins to auxins is also critical in determining shoot versus root formation. A high cytokinin to auxin ratio promotes shoot meristem formation, while callus or root meristems are formed when the cytokinin to auxin ratio is low (Lang and Kohlenbach, 1975). Generally, there are several types of PGRs, each having a well-defined effect on growth and development of the plant.

i) Effect of PGRs on Establishment of Callus and *In vitro* Shoot Induction in Jatropha

Soomro and Memon (2007) have reported the establishment of callus and suspension culture in Jatropha. In their study, excellent growth of callus (100%) was obtained in medium supplemented with 0.5mg/L 2, 4-D alone and with 2% v/v coconut milk in hypocotyl explants. Callus produced from hypocotyl explants grew faster during 7 to 30 days of culture then stabilized at a low growth rate. Calli cultured on this medium showed 8 fold increases in fresh weight by the fourth week of incubation. Callus was soft, friable, globular, lush green in color. Kumar *et al*. (2015) also reported that 100% callus formation from the media which supplemented with combination of NAA (5µM) and 2, 4-D (10µM) by using leaf lamina explants of Jatropha. This indicates that Jatropha is highly sensitive to Auxin with respect to the stimulation of cell division rather than the induction of roots (Shrivastava and Banerjee, 2008).

Sujatha and Mukta (1996) for the first time reported regeneration of Jatropha from explants such as hypocotyls, petiole, and leaf using a range of cytokinins like Zeatin, kinetin, BAP either singly or in combination of IBA. Independent of explants type, direct adventitious shoot bud induction was obtained highest on MS medium with 2.22 µM BA and 4.9 µM IBA. A combination of BAP and IBA along with growth additives (adenine sulphate, glutamine and L-arginine) was also found to be effective in inducing direct organogenesis from axillary node (Shrivastava and Banerje, 2008). Wei *et al.* (2004) developed *in vitro* culture of Jatropha on MS medium with IBA and BAP. In their study the highest regeneration frequency of adventitious buds were directly induced from the surface of epicotyl explants under the condition with the combinations of 0.1mg/l IBA and 0.5mg/l BAP. Weida *et al.* (2003) also

reported callus mediated shoot bud induction from Jatropha on MS medium supplemented with 0.5mg/l BAP and 1.0mg/l IBA.

Furthermore, the effectiveness of TDZ for inducing direct organogenesis of Jatropha from different explants also reported (Deore and Johnson, 2008; Kumar *et al.*, 2010; Sharma *et al.*, 2011). Khurana-kaul *et al.* (2010) reported that shoot bud induction frequency of TDZ was more than double as compared with BAP. Later on, ZhongGuang *et al.* (2012) had also reported callus mediated shoot bud induction using epicotyl explants in TDZ supplemented medium.

ii) Effect of PGRs on *In vitro* Root Induction of Jatropha

In vitro rooting of Jatropha was reported by many authors. The adventitious root induction in relation to micropropagation has great relevance and to induce rooting the explant is often grown aseptically in medium containing auxins such as IAA, IBA, or NAA (Sujatha and Mukta, 1996; Singh *et al*., 2010). Among various auxins used, IBA was shown to be effective in the rooting of Jatropha (Kochhar *et al*., 2005). Other studies confirm the root induction capacity of IBA albeit at different concentrations and with different efficiencies. IBA induced roots in 52% of the shoots within three weeks in medium containing 0.1-0.2mg/l IBA (Datta *et al*., 2007). 40% rooting was achieved with 0.1mg/l IBA after 5 weeks (Singh *et al*., 2010) and in 85.71% rooting induced from shoots on MS basal medium with 1mg/l IBA (Thepsamran *et al*., 2008). Kaewpoo and Techato (2009) reported a high rate of root induction frequency of 60% was obtained after 30-40days on MS medium supplemented with 0.5mg/l IBA. Deore and Johnson, (2008) also reported well-developed shoots were rooted on MS medium supplemented with 0.1mg/l IBA after 30 days.

On the other hand, rooting was comparatively better in half strength MS medium than full strength MS (Rajore and Batra, 2005; Shrivastava and Banerjee, 2008; Khurana-Kaul *et al*., 2010). This may be the use of half MS medium increases the C/N ratio by reducing the supply of nitrogen which is favorable to root formation (Medza Mve1 *et al.*, 2013).

Also micro shoots rooted well on full strength MS containing IBA (3.0 mg) and the plantlets successfully acclimatized in soil (Rajore and Batra, 2007). Rooting was effectively achieved on full strength MS supplemented with 1.0mg/l IAA within 6-7 days (Kalimuthu *et al*., 2007).

However, Rajore and Batra (2005) reported that i*n vitro* regenerated shoots induced roots on half strength MS medium with 3.0 mg IBA and 0.2% activated charcoal. They successfully transferred regenerated plantlets to field after initial acclimatization. Kumar and Reddy (2010) also maintained that half strength MS medium supplemented with 2% sucrose, 3 mg/l IBA in combination with 1mg/l IAA, 1mg/l NAA and 0.25mg/l activated charcoal found to be the best for promoting rooting. Later on, ZhongGuang *et al.* (2012) reported a high rate of root induction frequency of 90% was obtained on half medium supplemented with 0.1mg/l IBA. In general, rooting was achieved in the presence of IBA whereas IAA and NAA were less effective and often induces the formation of callus at the base of the shoot.

2.8. Stage of Micro Propagation

Micro propagation starts with the selection of plant tissues (explants) from a healthy, vigorous mother plant (Cassells and Doyle, 2005). Any part of the plant (leaf, apical meristem, bud and root) can be used as explants. The complete procedure of multiplication process usually involves a sequence of three or more stages, these are:

2.8.1. Stage 0: Preparation of Donor Plant

Any plant tissue can be introduced *in vitro*. To enhance the probability of success, the mother plant should be *ex vitro* cultivated under optimal conditions to minimize contamination in the *in vitro* culture (Cassells and Doyle, 2005).

2.8.2. Stage I: Initiation and Establishment of Aseptic Culture

The maintenance of aseptic (free from all microorganisms) or sterile conditions is essential for successful tissue culture procedures. In this stage, explants are surface sterilized and transferred into nutrient medium. Sterilization is the process of making explants contamination free before establishment of cultures. The surface sterilization of explants in chemical solutions is an important step to remove contaminants with minimal damage to plant cells (Garg *et al.*, 2014). Various sterilization agents are used to decontaminate the tissues. The most commonly used disinfectants are sodium hypochlorite (Tilkat *et al.,* 2009), Berekina (Tilahun *et al.,* 2013), calcium hypochlorite (Garcia *et al*., 1999), ethanol (Singh and Gurung, 2009) and mercuric chloride (Garg *et al.,* 2014). The collected explants after surface

sterilization is inoculated in the appropriate initiation medium under laminar air flow cabinet to achieve aseptic culture.

2.8.3. Stage II: Multiplication and Elongation

The aim of this phase is to increase the number of propagules (Saini and Jaiwal, 2002). The new outgrowths from the explants where dissected and transferred or sub-cultured to the multiplication medium under laminar air flow cabinet and where kept for observation till the plantlets multiply and elongate in mass.

2.8.4. Stage III: Rooting of Regenerated Shoots

The rooting stage may occur simultaneously in the same culture media used for multiplication of the explants. However, in some cases it is necessary to change media, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth. This stage basically involves rooting of individual shoots and pre-hardening of cultures to increase survival percentage (Hussain *et al*., 2012).

2.8.5. Stage IV: Acclimatization Stage

Acclimatization is the process by which an individual organism adjusts to changes in its environment. At this stage, the *in vitro* plants are weaned and hardened. Hardening is done gradually from high to low humidity and from low light intensity to high light intensity. Rooted micro shoots are removed from the culture vessel and the plants are then transferred to an appropriate substrate (sand, peat, compost, etc.) and gradually hardened under greenhouse. It is the prime measure of the survival rate of any tissue culture plantlets (Hussain *et al*., 2012).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The experiment of this study was conducted in the Plant Tissue Culture Laboratory at National Agricultural Biotechnology Research Center (NABRC), Holeta, during the period of 2017/2018. The center is located in the West Shewa Zone of Oromia Regional State, in Holeta town, 45 Km West of Addis Ababa at an altitude of 2400 meter above sea level, $9^{o}4'$ N latitude and $38^{o}30'$E longitude.

3.2. Planting Material

The seed of three Jatropha accessions were collected from Metema (Amhara region), Shewa Robit (Amhara region) and Adami Tulu (Oromia region) and used for these tissue culture experiments (Table 2). Those accessions were selected due to their best performance across the South Western region and Northern part of Ethiopia with respect to superior oil yield and quality, and resistance to drought, diseases and pests compared to other accessions tested**.** And also developing their varieties trials are under a progress at both of the centers, Wondo Genet Research Center and Sirinka Agricultural Research Center (Personal communication).

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

Note: WGRC= Wondo Genet Research Center; SARC= Sirinka Agricultural Research Center

The seeds were germinated on growth trays containing sterilized soil and kept in the greenhouse. They were watered thrice a week using a spraying can. After three weeks, the seed that germinated was transplanted into pot containing sterilized soil that has a combination of soil, sand and manure in the ratio of 2:1:1, respectively and kept as mother stockplant. The

plants were maintained in a greenhouse at Holeta Agricultural Research center, at average temperature of 25 ± 2 °C for about three months under natural sunlight conditions. The mother stockplant (Figure 2) was daily watered with tap water and also treated with Diazinon (60% Emulsifiable concentration) at 15 days interval to control pest infection. After three months of growth; very young, healthy and vigorous part of the plant (leaf and nodal) were collected and used as a source of explants to standardize the micropropagation protocol through direct and indirect shoot organogenesis.

a). Adami Tulu accession

b). Metema accession

c). Shewa Robit accession

Figure 2. Mother stock plant of Jatropha accession established from seed under greenhouse condition (three month old).

3.3. Preparation of Stock Solutions and Growth Media

3.3.1. Preparation of MS Stock Solution

Murashige and Skoog (1962) basal medium was used throughout this research activity. Initially, full strength stock solutions of macronutrients, micronutrients and vitamins and other organic supplements were separately prepared. To do so, appropriate amount of the powder form of each nutrient was weighted in grams per liter (Appendix Table 1) and dissolved in double distilled water. After all the components were completely dissolved using magnetic stirrer, the concentrated stock solutions were then stored in a refrigerator at a temperature of $+4^0$C and used for a maximum of one month.

3.3.2. Growth Regulators Stock Preparation

The PGRs used for the study were the cytokinin, 6- benzyl amimopurine (BAP), Kinetin (Kn) and Thidiazuron (TDZ) the auxins, 2, 4-dichlorophenoxyacetic acid (2,4-D), indole-3- butyric acid (IBA) and α-naphthalene acetic acid (NAA), and gibberrellin, gibberrellic acid (GA3). All growth regulator 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, 1N HCl and 99% ethanol based on the type of PGR (NaOH for auxins, HCl for cytokinin and ethanol for gibberellin). Then, the volume was adjusted by adding distilled water and mixed gently by using magnetic stirrer. Finally, growth regulators' stock solutions were stored in a refrigerator at +4°C for short term use. GA3 was filter-sterilized and added to the medium after autoclaving.

3.3.3. Growth Media Preparation

In this study, full strength MS basal medium were used for callus induction, shoot induction, multiplication, and elongation experiments whereas half strength MS medium (reducing the mineral salt concentrations) was used for *in vitro* root induction. The medium was fortified with 3% sucrose (w/v) and 0.25% (w/v) of Gerlite used as a gelling agent. The pH of the medium was adjusted to 5.8 (using 1N NaOH and 1N HCl) after addition of the growth regulators. The medium was dispensed in culture Jars (12x125 mm) containing about 40 ml of culture medium and autoclaved at 108Kpa and121^{o}C for 15 min. The sterilized media were

kept at room temperature (25ºC) for two days before culture to check any occurrence of microbial contamination.

3.4. Experimental Design and Treatments

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 and explants taken from different parts of the plant. Growth regulators were one factor and accessions were another factor. Each treatment had five replicates of culture Jars and set as experimental unit. All the external factors were held constant except the difference in the treatments. In the study three independent experiments were carried out as Experiment I: Surface sterilization of the Explant, Experiment II: Direct Organogenesis from nodal explant and Experiment III: Indirect Organogenesis from Leaf disc explant.

3.4.1. Experiment I: Surface Sterilization of Explant

Berekina (with 5% active ingredient of chlorine) was used for surface sterilization experiment for Jatropha leaf and nodal explants. These sterilants are preferred due to their availability and economic merits (Emongor *et al*., 2010; Tilahun *et al.*, 2013). In this experiment, Leaf and nodal explant (2^{nd} and 3^{rd} node from the apex) were collected from three months old healthy and vigorously growing Jatropha raised in the greenhouse. The excised explant materials were initially rinsed under tap water for 30 minutes to remove the dust particles from their surface. Then the explants were treated with commercial detergent (Largo, Ethiopia) for 5 minutes 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 in four different concentration of Local bleach (Berekina) (2.0, 2.5, 3.0, and 3.5% active ingredient of chlorine) for 5, 10, 15 and 20min time of exposure. After that the explants were rinsed with sterilized distilled water for three to four times to remove the residual effect of these sterilants. To facilitate the reaction two drops of 1mg/1ml of Tween - 20 was added into all the sterilant solutions prior to treatment. Then the treated explants were kept in sterilized distilled water for 10 min under aseptic condition. This is also to remove the residual portion

of the sterilants from the body of explants. After sterilization process was completed, individual leaf explant was trimmed aseptically into approximately 1cm^2 leaf disc segments and placed with the adaxial side in contact with the MS basal medium (Kumar *et al*., 2010a). And also the nodal segment was trimmed (l -l.5cm) with one node and inoculated by vertical orientation on the medium. Finally the effect of both concentration of Berekina and length of time exposure were determined for both explants from the culture on PGR-free MS basal media between two weeks intervals.

3.4.2. Experiment II: Direct Organogenesis from Nodal Explant

3.4.2.1. Culture Initiation

For culture initiation, single nodal explant (1-1.5cm) was inoculated on full strength MS media supplemented with different combinations of BAP (0, 1, 1.5, 2.0 mg/l) and IBA (0, 0.5, 1.0mg/l) along with Ascorbic acid (10mg/l) for prevention of browning of cultures. Four 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 three weeks.

3.4.2.2. 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, 1.0 mg/l). Both types of cytokinins (BAP and Kn) are used in this study since synergetic effect of BAP and Kn combination on shoot multiplication of many plants were reported by previous studies (Madhulatha *et al.*, 2004; Tilahun *et al.*, 2014). Five shoots per culture jars and five replications for each treatment were used. The cultures were maintained at 25 ± 2°C with a 16 hour 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.

To achieve shoot elongation, the multiplied shoots were transferred onto growth regulators free MS basal medium for two weeks. At the same time this procedure also used to avoid the carry over effect of multiplication media on *in vitro* rooting (Tilahun *et al.*, 2014).

3.4.2.3. Root Induction

The elongated shoots with three to four leaves were excised and cultured on half strength MS media supplemented with different 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 ± 2^0C and 16 hour photoperiod provided by white florescent lamps. Each root growth parameters were recorded within four weeks of the culture.

3.4.3. Experiment III: Indirect Organogenesis from Leaf disc Explant

3.4.3.1. Callus Induction

After sterilization, about $1cm^2$ leaf disc of the three Jatropha accessions (Metema, Adami Tulu and Shewa Robit accession) were transferred to callus induction media consisting of MS basal medium supplemented with various combinations of 2, 4-D (0, 0.5, 1.0, 1.5 mg/l) and BAP (0, 1.0, 1.5, 2.0 mg/l). Five leaf disc explants per jar were used and each treatment was replicated five times. All the cultures were incubated at 25 ± 2°C in darkness to promote callus formation and discourage greening of the callus. The percentage proportion of callus induction on leaf discs was evaluated at an interval of four weeks after inoculation of explant.

3.4.3.2. Shoot Proliferation from Callus

Well-established organogenic callus (4-week-old) grown on MS medium supplemented with combination of BAP (1.0 mg/l) and 2,4-D (1.0 mg/l) for all accessions were used. About 0.5gram of calli were transferred to shoot regeneration media, consisting of MS basal medium containing various combinations of cytokinins viz. BAP (0, 0.5, 1.0 mg/l) and TDZ (0, 0.25, 0.5, 1.0 mg/l) individually and in combination with different concentrations of IBA (0, 0.1, 0.2 mg/l). There were three callus clumps per jar and five replicates per treatment. Then the culture was maintained in a growth room at a temperature of 25 ± 2^0C and 16 hour photoperiod provided by white florescent lamps. The cultures were subcultured once transferring into fresh

media after three weeks for further initiation of adventitious shoots. During subculture removal of dead, dark brown cells was done. The percentage of shoot organogenesis and number of adventitious shoot initiated from callus was recorded after six weeks of transferring the callus on shoot regeneration media.

3.4.3.3. Multiplication, Elongation and Rooting of Shoots Derived from Callus

Multiplication and *in vitro* rooting of shoots which derived from callus was also performed accordingly with those derived from the direct organogenesis experiments (see section 3.3.4.2 and 3.3.4.3). However to achieve shoot elongation, the highest multiple shoot clusters were dissected into smaller sizes and transferred onto fresh medium which contains 0.5mg/l BAP and 1.0mg/l GA3 for two weeks according to the protocol of Jeevan *et al*. (2013). We added the active Gibberellin form (GA3) to the media since the promotive effect of GA3 on elongation of stunted shoots generated has been reported also in several other plant species (Geetha *et al.*1998; Purohit and Singhvi, 1998). Then the elongated shoots were transferred to PGRs free medium for two weeks to reduce latent effects of the previous medium composition (Tilahun *et al.*, 2014).

3.5. Growth conditions

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

3.6. 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 transferred to 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.

3.7. Data Recorded

The following plant growth variables were recorded from this experiment:

Percentage of contamination of the explant: Contamination rate was determined by counting the number of explant contaminated during sterilization protocol in comparison to the total number of explants inoculated after fifteen days of culturing explants.

Callus forming explants (%): Percentage of callus response was recorded from the explants.

$$\textbf{\% of callus response} = \frac{\text{Number of explant induce callus}}{\text{Total number of explant inoculated}} \text{X100}$$

Days to callus formation: The treatments were observed daily and noted for calli formation around the leaf edges. Days to callus formation were derived by the difference between date of first callus formation and date when leaf explant was transferred to callus induction media.

Callus fresh weight (g): the average callus fresh weights were recorded at the end of the initiation phases in callus induction medium by using an electronic sensitive balance.

Number of adventitious shoots per clump of callus: The average number of adventitious shoots regenerated from each callus

Days to shoot induction: The treatments were observed daily and the number of days taken to shoot initiation was recorded by counting the number of days from date of inoculation of explant until shoot initiation and averaged.

Percent of shoot induction: - was calculated using the following formula:

% of shoot induction = $\frac{\Sigma xi}{n}$* 100, where, xi = Number of explants with shoots; n= Number of inoculated explants/callus

Number of shoots per explants: is the average number of dissectible shoots regenerated from each cultured explants.

Length of shoot (cm): is the average length of shoot developed from explants. It was measured from base to the tip of the plantlet by using well sterilized ruler.

Number of leaves per shoot: Average number of leaves per plant was determined by counting the number of fully developed leaves formed in each plant and then determining the mean number of leaves per plant for each treatment.

Percent of root induction:- was calculated using the following formula:

% of root induction= $\frac{\Sigma xi}{n}$* 100, where, xi = Number of shoots with roots; n= Number of transferred shoot

Number of days to root induction: The duration of time in days taken for induction of root from the day of culturing.

Number of root per shootlet: is the average number of roots was determined by counting the number of roots formed in each plant and then determining the mean number of roots for that treatment.

Length of root (cm): is the average length of root developed from the shoot. It was measured from the collar region to the highest root tip by using sterilized ruler.

Survival rate: It was the competence or the ability of the *in vitro* derived plantlets to endure in the *ex vitro* condition after one month of acclimatization in the green house.

3.8. Data Analysis

Treatment effects in all experiments were determined by using the Proc. mixed model analysis of variance (ANOVA) and significant differences among treatments were determined by Fisher's Least Significance Difference (LSD) using the SAS software package version 9.0 (SAS, 2002). For all the data analysis, probability level at 5% was considered for statistical significance.

4. RESULT AND DISCUSSION

Organogenesis is a complex process and it depends upon the addition of exogenous plant growth regulators in the media to make the explant organogenic competent for differentiation, which varies from species to species and parts of plants taken as explant. In the present study, plant regeneration via direct and indirect organogenesis of three Ethiopian Jatropha accessions (Metema, Adami Tulu and Shewa Robit accession) were investigated. Results obtained from experiments on *in vitro* callus induction, shoot induction, rooting of shoots and acclimatization of plantlets are presented and discussed in the following sections.

4.1. Surface Sterilization of Explants

In the present study, Berekina (with 5% active ingredient of chlorine) was successfully used for sterilization protocol for Jatropha leaf and nodal explants. Analysis of variance (ANOVA) revealed that the concentration of Berekina, duration of explants' exposure to sterilant and interaction of Berekina with time had highly significant effect ($P<0.01$) on the level of contamination and plant tissue death. There was also highly significant effect ($P<0.01$) between the two explants (Appendix Table 2).

The highest percentage of clean and healthy survived explants (86.16%) were recorded when the leaf explants were treated with 2.5% of Berekina for 15 minutes. In the case of nodal explants, the highest percentage of clean explants (80%) was observed when the explant treated with 3.0% of Berekina for 15 minutes. Whereas, the lowest percentage of surviving of explants were recorded for leaf explants (18.16%) at 20 minutes exposure and 3.5% of Berekina and (19.89%) were obtained from nodal explant when it was surface sterilized with 2% of Berekina for 5 minutes (Table 3). In this study, the lowest concentrations (2%) of Berekina at 5 and 10 minutes resulted in low percentages of survived explants for both leaf and nodal explants. This might be due to the insufficiency of the concentration of Berekina and length of exposure time to kill culture contaminants. Similarly, among the given concentrations of Berekina the highest concentrations (3.5% of Berekina) at longer time (20 min) of exposure gave low percentages of survived plantlets for both explants. This might be due to the high bleaching activity of chlorine which killed the cells. The current result is consistent with the findings of Oyebanji *et al.* (2009) and Tilahun *et al.* (2013) who reported

that explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period.

Statistical analysis also revealed that there were significant differences between the two tested types of explants of Jatropha. In this case, the plausible cause may be due to the fact that different explants need different level of concentration for sterilization. The finding is in line with a study by Colgecen *et al.* (2011) which found that requirements on the concentration and time of exposure differ from one plant to another and for different parts of plants depending on their morphological characters like softness/ hardness of the tissue.

Table 2. Effect of different concentrations of surface sterilant of Berekina (with 5% active ingredient of chlorine) and time exposure on survival of aseptic of leaf and nodal explants of Jatropha. *Means with different letter in a column and rows are statistically significant at P-values < 0.05. The values are mean ± SE (where, n= 3).*

Berekina (%)	**Time of exposure (Minutes)**	**Survived aseptic explants (%)**	
		Leaf	**Nodal**
0	0	0.00 ±0.00	0.00 ±0.00
2.0	5	23.89 ±0.67^{qr}	19.89 ±0.48^{rs}
2.0	10	36.66 ±1.66^{mn}	31.66 ±1.66^{op}
2.0	15	52.89 ±1.49^{ij}	42.53 ±1.44^{kl}
2.0	20	38.33 ±1.66^{lm}	41.16 ±1.96^{kl}
2.5	5	34.50 ±2.46^{mno}	25.00 ±0.00^{q}
2.5	10	72.33 ±1.45^{de}	52.66 ±2.33^{ij}
2.5	15	86.16 ±1.16^{a}	73.11 ±0.98^{cde}
2.5	20	50.00 ±0.00^{j}	58.33 ±1.66^{gh}
3.0	5	53.11 ±1.60^{ij}	44.50 ±2.46^{k}
3.0	10	75.00 ±0.00^{cd}	76.66 ±1.66^{bc}
3.0	15	56.83 ±1.58^{hi}	80.00 ±0.00^{b}
3.0	20	29.55 ±2.51^{p}	58.33 ±1.66^{gh}
3.5	5	61.60 ±0.00^{fg}	55.83 ±0.83^{hi}
3.5	10	41.66 ±1.66^{kl}	63.61 ±1.36^{f}
3.5	15	33.33 ±1.66^{nop}	69.11 ±0.89^{e}
3.5	20	18.16 ±1.16^{s}	21.16 ±1.96^{qrs}
CV (%)	**5.31**		
LSD (5%)	**4.25**		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n= number of samples

4.2. Shoot Regeneration through Direct Organogenesis

4.2.1. Shoot Induction from Nodal Explants

4.2.1.1. Days to Shoot Induction

Days required for shoot induction was highly significantly ($P<0.01$) influenced by the main effect of growth regulator combinations and concentrations, however the accessions and interaction effect of the two factors is non-significant ($P>0.05$) on this parameter (Appendix Table 3).

Considerable variation was observed in days to shoot induction regarding to explant cultured on different concentration of growth regulators. The minimum number of days (9.27 days) to shoot induction was recorded for explants cultured on MS media supplemented with combination of 1mg/l BAP and 0.5mg/l IBA followed by (9.8 days) on MS media supplemented with 2.0 mg/l BAP. Whereas, the maximum mean days (17.20 days) to shoot induction was recorded for explants cultured on the media supplemented with combination of 1mg/l BAP and 1mg/l of IBA (Table 4). The result of this study revealed that the number of days to shoot emergence increased with increased in concentration of IBA in the treatments. This might be due to the callus formation under the base of the explant at higher concentration of IBA. Kalimuthu *et al.* (2007) also found that higher concentration of auxins is generally inhibitory to morphogenesis and substitution of these reagents with an appropriate auxin-cytokinin ratio is essential to obtain proper shoot primordial. This proves the fact that the cross-talk between auxin and cytokinin during *in vitro* organogenesis is very important in achieving easy and stable establishment of the explants. Studies on the classical chemical regulation of growth and organ formation from *in vitro* plant tissues have indicated that excess of cytokinin over auxin promotes shoot formation, whereas a low ratio of cytokinin to auxins promotes callus induction (Skoog and Miller, 1957).

Table 3. Effects of PGRs (BAP and IBA) on Days to shoot initiation of *in vitro* nodal culture of Jatropha. *Means with different letter in a column are statistically significant at P-values < 0.05. The values are mean ± SE (where, n=15).*

PGR concentration (mg/l)		
BAP	**IBA**	**Days to shoot initiation**
0	**0**	-
0	**0.5**	16.40±0.63^{b}
0	**1.0**	-
1.0	**0**	13.87±0.13^{d}
1.0	**0.5**	9.27±0.21^{h}
1.0	**1.0**	17.20±0.11^{a}
1.5	**0**	13.07±0.15^{e}
1.5	**0.5**	12.00±0.09^{f}
1.5	**1.0**	14.33±0.16^{c}
2.0	**0**	9.80±0.17^{g}
2.0	**0.5**	12.87±0.13^{e}
2.0	**1.0**	16.27±0.18^{b}
LSD (5%)		**0.33**
CV (%)		**4.11**

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n= number of samples, '-' indicates no response

4.2.1.2. Percentage of 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 (Appendix Table 3).

The highest percentage (90%) shoot induction was recorded for both Shewa Robit and Metema accessions on MS media supplemented with combination of 1mg/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 combination of 1mg/l of BAP and 1mg/l IBA for all accessions (Table 5). 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 also obtained on MS medium which contains only BAP (2mg/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 8.0μM 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 (Fig. 3D). 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).

Table 4. 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. *Different letters (within columns and rows) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).*

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**		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n= number of samples, '-' indicates no response

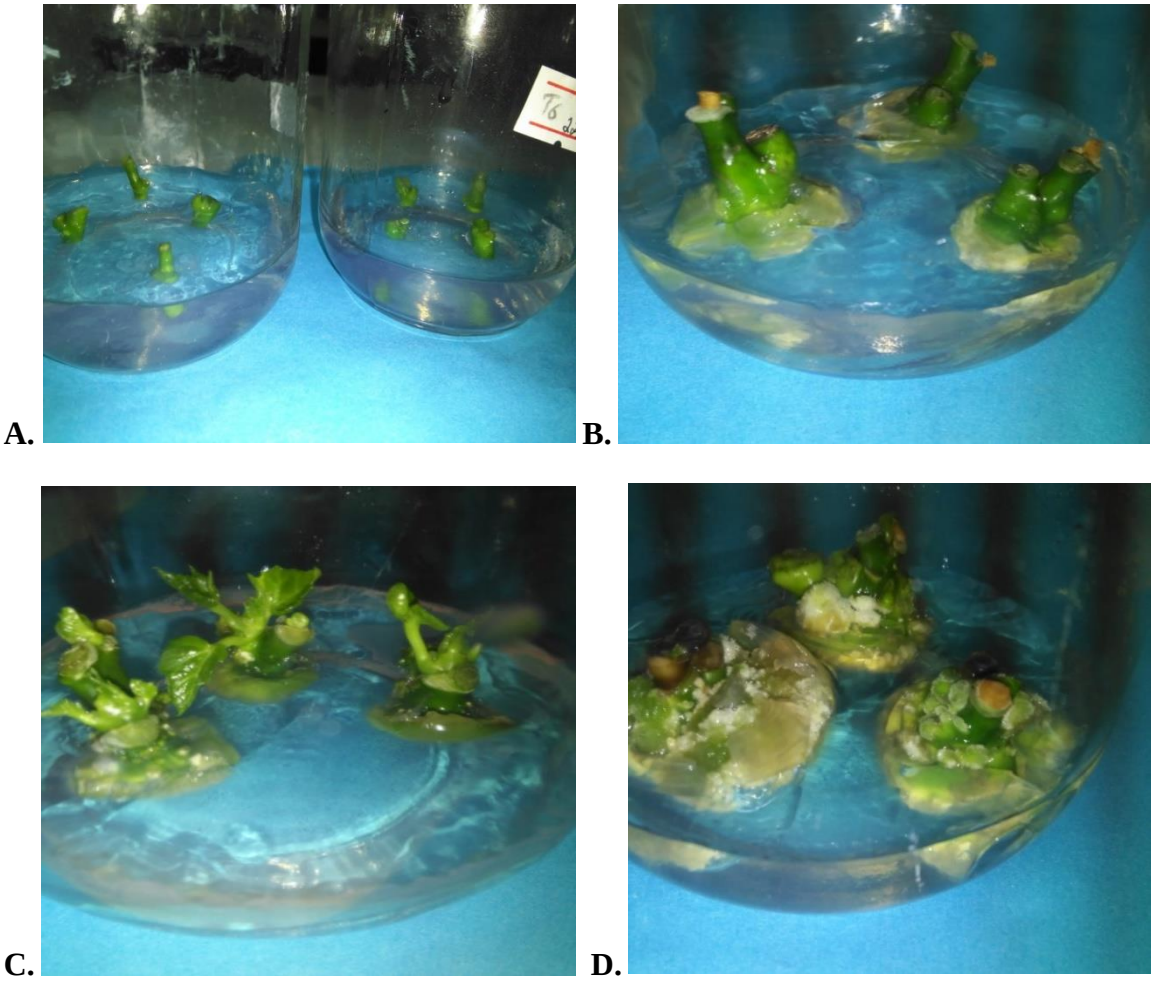

Figure 3. Effect of BAP and IBA on *in vitro* shoot induction of nodal cultures of Jatropha accessions. A) Nodal explants cultured on initiation media at day one; B) Nodal explant cultured on PGRs free MS medium after three weeks; C) Shoot initiated at 1.5mg/l BAP and 0.5 mg/l IBA from Nodal culture after three weeks and D) Explant cultured on MS medium containing 1mg/l of IBA forming strong callus.

4.2.2. Shoot Multiplication

In the present study, full strength MS medium containing different combinations and concentrations of cytokinins (BAP and Kn) have been used to assess the multiplication potential of the already initiated shoots of the three Jatropha accessions.

4.2.2.1. Number of Shoot per Explant

The number of shoots proliferated per explants were highly significantly ($P<0.01$) influenced due to main effects of the two factors and also there was significant difference ($P<0.05$) on this parameter due to interaction effects of the two factors (Appendix Table 4).

The maximum number of shoots was recorded for Metema (6 shoots) on MS media supplemented with combination of 0.5mg/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 6). In this study, the optimum combination of BAP and Kn which was 0.5mg/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.

4.2.2.2. Shoot Length

The statistical analysis revealed that there is significant difference on length of shoot per explant due to the main effects of growth regulators, accessions and interaction effect of the two factors (Appendix Table 4).

The maximum shoot length was recorded for Shewa Robit (3.2cm) on media supplemented with 0.5 of mg/l Kinetin. Whereas, the lowest mean shoot length (1.8-1.84cm) was recorded for all the three accession shoots developed on media supplemented with 1.5 mg/l BAP (Table 6). 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 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.

Table 5. Effect of different concentration and combination of BAP and Kn on number of shoots and shoot length of three Jatropha accessions. *Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).*

Jatropha Accessions	Cytokinin (mg/l)		No of shoot per explant	Length of shoot(cm)
Metema	**BAP**	**Kn**		
	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**

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error

4.2.2.3. Number of Leaf per Shoot

There was a highly significant difference ($P<0.01$) for the main effects of plant growth regulator combinations and concentrations on number of leaves developed per shoot; however the main effect of accession and the interaction effect of the two factors had no significant influence on number of leaf per explants (Appendix Table 4).

The maximum mean number of leaf (7 per shoot) was recorded on MS media supplemented with 0.5mg/l of BAP followed by (6.43 per shoot) on media supplemented with combination of 0.5mg/l of BAP and 0.5mg/l of Kn. Whereas, the lowest mean number of leaf (2.97 per shoot) was recorded for shoots induced on MS media supplemented with 1.5mg/l of BAP (Table 7). 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 (0.5mg/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 1mg/l BAP and 1mg/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 growth room.

Table 6. Main effect of the different concentrations and combinations of Cytokinins (BAP and Kn) on number of Leaves per shoot of Jatropha accessions. *Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15).*

PGR concentration (mg/l)		
BAP	**Kinetin(Kn)**	**Number of Leaf per shoot**
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**

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples


A.


B.


C.


D.


E.

Figure 4. Effects of BAP and Kn on Shoot multiplication of Jatropha accession after four weeks of culturing. A) Shoots initiated for multiplication on 0.5mg/l BAP; B) Multiplied shoots of Metema accession at 0.5mg/l BAP; C) Length of Shewa Robit shoots at 0.5mg/l Kn; D; Length of Metema shoots at 0.5mg/l Kn; E) Length of Adami Tulu shoots at 0.5mg/l Kn.

4.2.3. Rooting Induction

4.2.3.1. Number of Days to Root Induction

Days required for root induction were highly significantly ($P<0.01$) affected by the main effect of growth regulator concentrations and combinations. However there was no significant difference ($P>0.05$) among accessions and interaction effect of the two factors on days taken to induce roots for the cultured shoots (Appendix Table 5).

The minimum number of days to root induction (15 days) was recorded on half MS media supplemented with 0.25 mg/l of IBA followed by (15.5 days) on media supplemented with combination of 0.25 mg/l of IBA and 0.25 mg/l of NAA. Whereas, the maximum mean days to root induction (20.53 days) was recorded on half MS media supplemented with combination of 1mg/l of IBA and 0.5 mg/l NAA (Table 8). From this result, it can be depicted that as the concentration of IBA alone and combination with NAA increased on half MS medium, the number of days to root induction also increased. This shows that the increase in concentration of auxin beyond the level required to initiate rooting is unnecessary or detrimental to the shoot end cells because of the toxicity effects. Besides increasing the IBA or NAA level beyond optimum level to would result in increased callusing which would in turn deter the induction of roots and associated root growth and development (Shrivastava and Banerjee, 2008; Maharana *et al.*, 2012). In similar to the present study, Datta *et al.* (2007) reported that root formation occurred within three weeks in half MS medium containing 0.1-0.2 mg/l IBA for Jatropha. However this result is in contrast to those of Kaewpoo and Te-chato, (2009) who reported that Jatropha regenerated shoots were rooted on full MS medium supplemented with 0.5 mg/l IBA after 30–40 days of culture. The reason for requirement of long period of time to root induction in their study might be due to composition media they have used, since reducing the mineral salt concentrations within the full MS basal medium to half had also been reported to enhance the root initiation, as well as other associated root growth and development (George *et al.*,2008).

4.2.3.2. Rooting Percentage

The percentage of rooting was highly significantly (P<0.01) influenced by PGRs combinations and concentrations, accessions and the interaction effect of the two factors (Appendix Table 5).

The highest rooting percentage (88%) was recorded for Shewa Robit accession on media supplemented with 0.25mg/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 1mg/l IBA and 0.5mg/l NAA (Table 9). 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 (Fig. 5D). 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.

4.2.3.3. Number of Roots Induced per Shoot

The result showed 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) (Appendix Table 5).

The maximum root number (5.43) was recorded on MS media supplemented with 0.25mg/l of IBA followed by (4.8) on media supplemented with combination of 0.5mg/l of IBA and 0.25mg/l of NAA (Table 8). 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 0.2mg/l IBA for best rooting in Jatropha. However, there are other studies who recommended 3mg/l IBA addition to half MS medium for best rooting in Jatropha (Shrevastava 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).

4.2.3.4. Length of Root induced per shoot.

The mean length of roots induced on half MS media was highly significant ($P<0.01$) different due to the individual main effects of different concentrations and combinations of PGRs and Jatropha accessions. The statistical analysis also showed that there was significant difference ($P<0.05$) due to interaction effects of the two factors on length of roots (Appendix Table 5).

The maximum mean root length was obtained for Shewa Robit (4.3cm) on the media supplemented with 0.25mg/l IBA followed by (4cm) and (3.8cm) 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 9). In this study, the optimum level of IBA concentration is 0.25mg/l for all the three accessions. Datta *et al.* (2007) have also reported that half MS medium fortified with 0.2mg/l regenerated better root length (8.7cm) 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).

Table 7. Number of days to root induction and number of roots per shoots as affected by different concentration and combination of Auxins (IBA and NAA). *Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15).*

PGR concentration (mg/l)		**Number of days to root induction**	**Number of roots per shoot**
IBA	**NAA**		
0	**0**	17.00±0.09^{f}	1.80±0.14^{i}
0	**0.25**	-	-
0	**0.5**	-	-
0.25	**0**	15.07±0.12^{j}	5.43±0.08^{a}
0.25	**0.25**	15.50±0.12^{i}	4.07±0.12^{d}
0.25	**0.5**	15.93±0.12^{h}	3.31±0.06^{e}
0.5	**0**	16.67±0.12^{g}	4.53±0.08^{c}
0.5	**0.25**	17.83±0.09^{e}	4.80±0.11^{b}
0.5	**0.5**	18.20±0.14^{d}	2.87±0.13^{f}
1.0	**0**	19.00±0.00^{c}	3.50±0.05^{e}
1.0	**0.25**	20.20±0.11^{b}	2.47±0.06^{g}
1.0	**0.5**	20.53±0.13^{a}	2.07±0.12^{h}
LSD (5%)		**0.29**	**0.25**
CV (%)		**2.82**	**12.28**

LSD=Least Significant Difference, CV= Coefficient of variation, SD= Standard Error and n=number of samples, '-' indicates no response

Table 8. Effects of different concentration and combination of IBA and NAA on rooting percentage and root length of three Jatropha accessions. *Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).*

Jatropha Accessions	**Auxin (mg/l)**		**Rooting response (%)**	**Root length (cm)**
Metema	**IBA**	**NAA**		
	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**

CV= Coefficient of variation, LSD=Least Significant Difference, SD= Standard Error

A.  **B.** 

C.  D. 

Figure 5. Effects of IBA and NAA on root formation of three Jatropha accessions after four weeks. A) Roots of Shewa Robit accession cultured on 0.25mg/l IBA; B) Roots of Adami Tulu accession cultured on 0.25mg/l IBA; C) Roots of Metema accession cultured on 0.25mg/l IBA; and D) Callus formation on rooting MS media with 0.5mg/l NAA.

4.3. Shoot Regeneration through Indirect Organogenesis

4.3.1. Callus Induction

In the present study, leaf discs from three Jatropha accessions placed on the induction medium enlarged and exhibited varied response depending on the media used for culturing. Its enlargement and swelling was observed after seven days of inoculation. Explants cultured in absence of PGRs senesced without producing callus (Fig. 6B). Well-developed calli were observed in all three accessions after four weeks of culturing. The texture of the callus was a compact form for all treatments with white or white-yellow color.

4.3.1.1. Days to Callus Formation

The analysis of variance (ANOVA) showed that there is highly significant ($P<0.01$) difference due to the main effect of PGRs combinations and concentrations on mean number of days taken to form calli formation. However, there were no significant ($P>0.05$) effects recorded due to difference in accession and the interaction effect of PGRs and accessions (Appendix Table 6).

The minimum number of days (13.6 days) to callus induction was recorded for explants cultured on MS media supplemented with combination of 1mg/l BAP and 1mg/l 2,4-D followed by (14 days) on MS media supplemented with 1.5mg/l BAP and 1mg/l 2,4-D. Whereas, the longest time (18.87 days) taken to form callus was observed when callus induction medium was supplemented with combination of 2mg/l BAP and 0.5mg/l of 2,4-D (Table 11). From these results, increasing BAP levels in media within constant rate of 2,4-D led to an increase in the mean days taken to form calli. The media which supplemented with BAP alone did not induce a callus. This indicated that presence of auxins in the media play a crucial role in the callus formation of Jatropha explants *in vitro* (Rajore and Batra, 2007; Kumar *et al*., 2008; Kumar *et al.*, 2015). The result of this study also revealed that the number of days to callus emergence decreased with increased in concentration of 2,4-D (0.5-1mg/l) in the treatments. Kumar *et al.* (2015) also reported that leaf explants Jatropha started callusing within two weeks on the media containing 2, 4-D (10µM). However, further increasing 2,4-D levels in media led to an increase in the mean days taken to form calli. Radhakrishnan *et al*.

(2001) reported that the cells used up 2,4-D as required and any excess began to actively show the herbicidal effects that therefore slowed down the callus induction process.

4.3.1.2. Percentage of Callus Formation

Significant differences for percentage of callus induction were observed among the PGRs combinations and concentrations as well as interaction of PGRs with accessions. However, the percentage of callus induction was not significantly ($P>0.05$) different among the accessions (Appendix Table 6).

The best callus formation (100%) was observed when MS medium was supplemented with combination of BAP (1.0 mg/l) and 2, 4-D (1.0 mg/l) for all accessions. Whereas, the lowest percentage (47.8-48.6%) of callus formation was recorded for all the three accessions explants cultured on media supplemented with 2.0mg/l BAP and 1.5mg/l 2,4-D (Table 10). This indicated that callus induction frequency decreases with further increasing the concentration of both 2,4-D and BAP. Several authors reported that appropriate concentrations and combinations of cytokinins and auxins are important to produce Jatropha callus (Costa *et al.*, 2015; Kumar *et al*., 2015). On the other hand, the MS media containing only 2,4-D, even at a low concentration resulted in a better callus formation (Fig. 6C). Similar observation was also made by Thao *et al*. (2003) and Soomro and Memon (2007) concluding that 2,4-D was prerequisite for callus formation in many of plant species. Meanwhile, no callus formation was observed and the explants only showed leaf expansion on medium containing BAP alone (Fig. 6D). These results are supported by Kaewpoo and Techato, (2009) who used different concentration of BAP (1mg/l, 2mg/l and 3mg/l) to induce callus from embryo cultures of Jatropha. The authors finding report showed that BAP alone has induced shoot rather than callus. It has long been suggested that strong auxins such as 2,4-D are mainly efficient in promoting cell clumping and further developing of the callus.

4.3.1.3. Callus Fresh Weight

There is highly significant ($P<0.01$) difference due to the main effect of PGRs combinations and concentrations on callus fresh weight. However, there were no significant ($P>0.05$) effects recorded due to difference in accessions and the interaction effect of PGRs and accessions (Appendix Table 6).

The highest calli weight (2.23g) was recorded on MS media supplemented with combination of 1.0mg/l of BAP and 1.0mg/l 2,4-D followed by (2.06g) on media with 1.5mg/l of BAP and 1.0mg/l 2,4-D (Table 11). Whereas, the lowest overall mean calli weight (0.98g) were observed for explant induced on MS media containing high levels of BAP (2.0mg/l) and 2,4-D (1.5mg/l) were used. This results confirmed that despite 2,4-D being an effective auxin in producing callus in Jatropha, it was active for callus induction when used in small amounts (Soomro and Memon ,2007). Radhakrishnan *et al*. (2001) also reported that high 2,4-D concentration has been shown to have herbicidal effects on plants causing cell growth inhibition and at highest cell death.

Table 9. Interaction effect of PGRs (BAP and 2,4-D) combination with different Jatropha accessions on callus formation percentage of explants excised from leaf after 30 days of culturing. *Different letters (within columns and rows) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).*

PGRs Concentration (mg/l)		**Callus formation (%)**		
BAP	**2,4-D**	**Metema**	**Adami Tulu**	**Shewa Robit**
0	**0**	-	-	-
1.0	**0**	-	-	-
1.5	**0**	-	-	-
2.0	**0**	-	-	-
0	**0.5**	82.00 ±0.00^{fg}	84.00 ±0.00 ^{f}	84.00 ±0.00^{f}
1.0	**0.5**	52.20 ±0.80^{kl}	52.00 ±0.00^{kl}	53.20 ±0.20^{k}
1.5	**0.5**	60.00 ±0.00^{i}	61.20 ±0.20^{i}	60.20 ±0.20^{i}
2.0	**0.5**	48.80 ±0.49^{mn}	49.20 ±0.49^{mn}	50.00 ±0.00^{m}
0	**1.0**	82.60 ±0.60^{f}	80.60 ±0.20^{g}	81.00 ±0.00^{g}
1.0	**1.0**	100.00 ±0.00^{a}	100.00±0.00^{a}	100.00 ±0.00^{a}
1.5	**1.0**	95.60 ±0.40^{b}	93.20 ±0.49^{c}	94.80 ±0.8^{b}
2.0	**1.0**	75.20 ±0.20^{h}	75.00 ±0.00^{h}	75.60 ±0.60^{h}
0	**1.5**	58.00 ±1.22^{j}	57.00 ±1.22^{j}	57.60 ±1.12^{j}
1.0	**1.5**	87.20 ±0.49^{e}	86.40 ±0.40^{e}	88.80 ±0.49^{d}
1.5	**1.5**	52.00 ±1.22^{kl}	52.00 ±1.22^{kl}	51.60 ±0.40^{l}
2.0	**1.5**	48.60±0.97^{mn}	48.00 ±0.00^{n}	47.80 ±0.80^{n}
CV (%)		**2.19**		
LSD (5%)		**1.43**		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples, '-' indicates no response

Table 10. Main effect of PGRs combinations and concentrations on days to callus formation and callus fresh weight of leaf explants of Jatropha. *Different letters (within columns) indicate significant differences at P-values <0.05. The values are mean ± SE (where, n=15).*

PGRs concentration (mg/l)			
BAP	**2,4-D**	**Days to callus formation**	**Fresh weight of callus(g)**
0	**0**	-	-
1.0	**0**	-	-
1.5	**0**	-	-
2.0	**0**	-	-
0	**0.5**	15.67 ± 0.13^{f}	1.44 ± 0.004^{h}
1.0	**0.5**	17.00 ± 0.00^{d}	1.27 ± 0.003^{j}
1.5	**0.5**	17.53 ± 0.13^{c}	1.43 ± 0.015^{h}
2.0	**0.5**	18.87 ± 0.13^{a}	1.32 ± 0.008^{i}
0	**1.0**	15.03 ± 0.12^{g}	1.97 ± 0.001^{c}
1.0	**1.0**	13.60 ± 0.16^{i}	2.23 ± 0.004^{a}
1.5	**1.0**	14.07 ± 0.07^{h}	2.06 ± 0.005^{b}
2.0	**1.0**	16.47 ± 0.19^{e}	1.55 ± 0.003^{f}
0	**1.5**	16.87 ± 0.09^{d}	1.48 ± 0.002^{g}
1.0	**1.5**	14.93 ± 0.12^{g}	1.75 ± 0.009^{d}
1.5	**1.5**	17.93 ± 0.12^{b}	1.71 ± 0.004^{e}
2.0	**1.5**	18.00 ± 0.00^{b}	0.98 ± 0.001^{k}
LSD (5%)		**0.29**	**0.015**
CV (%)		**3.32**	**1.75**

CV= Coefficient of variation, LSD= Least Significant Difference, SE= Standard Error, n=number of samples, '-' indicates no response

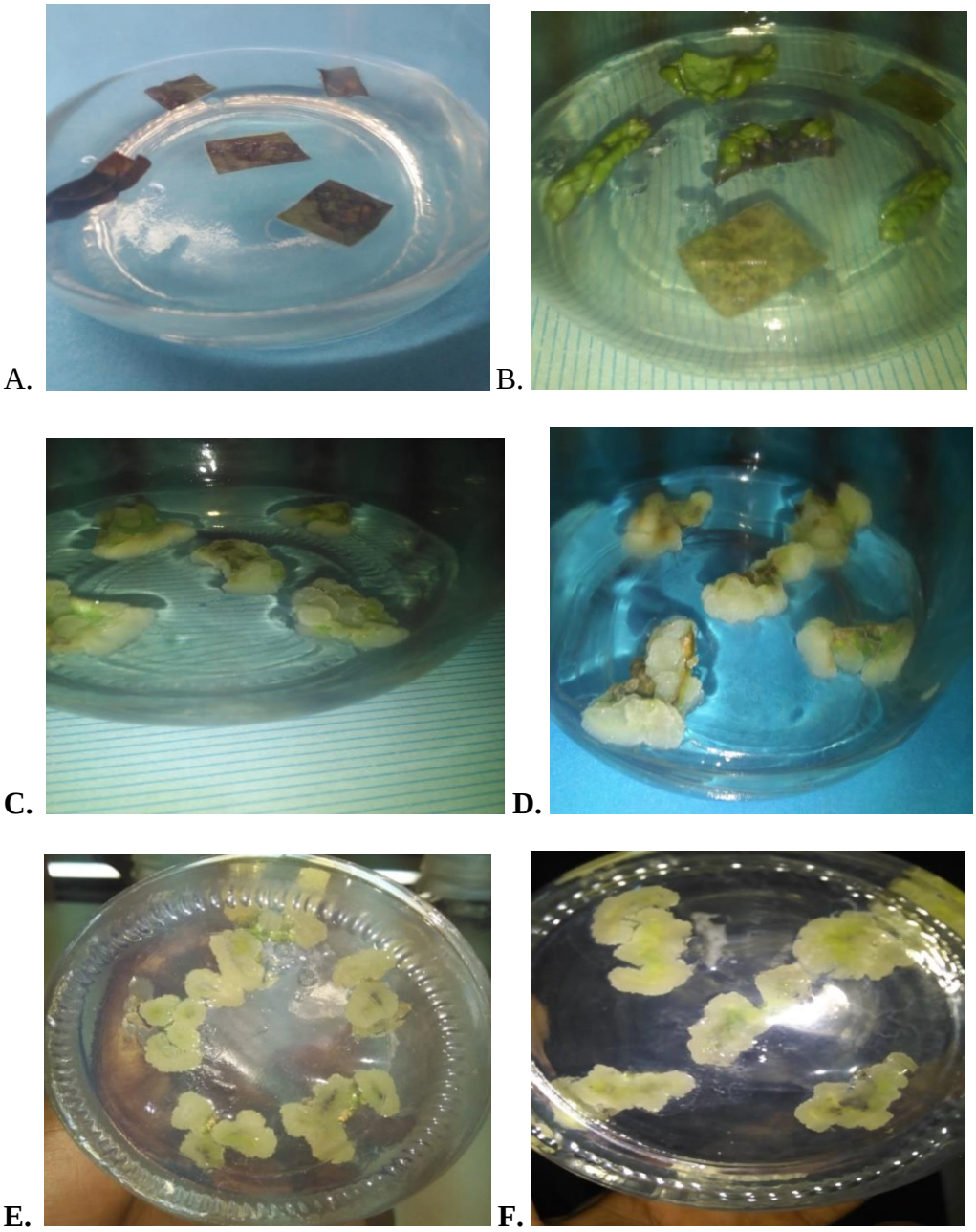

Figure 6. Callus induction after four weeks of culture in different combination of BAP and 2, 4-D for the three Jatropha accessions. A) Explants cultured on hormone free MS medium; B) Explants cultured on medium containing of BAP alone; C) Callus induction by 2,4-D (0.5mg/l) only; D) Leaf disc explants of Metema cultured on a medium containing 1mg/l BAP +1mg/l 2,4-D; E) Leaf disc explants of Shewa Robit accession cultured on a medium containing 1mg/l BAP+1mg/l 2,4-D and F) Leaf disc explants of Adami Tulu accession cultured on a medium containing 1mg/l BAP +1mg/l 2,4-D.

4.3.2. Adventitious Shoot Proliferation from Callus

Regenerating shoots from callus in plants is a key step in achieving whole plant transformation. Hence, in the present study in order to regenerate shoots from callus, healthy and fresh calli from optimal media concentration was transferred to MS solid media supplemented with either various concentrations of BAP or TDZ combined with different concentrations of IBA.

4.3.2.1. Percentage of Adventitious Shoots Formation

The percentage of shoot regeneration was significantly ($P<0.01$) affected by the type and concentration of PGRs, accessions and interaction effect of the two factors (Appendix Table 7)

The highest percentage of shoot regeneration from callus (66.67%) was observed for Shewa Robit in MS medium supplemented with combination of 0.5mg/l TDZ and 0.1 mg/l IBA followed by 64% and 61.33% for Metema and Adami Tulu accessions, respectively, on the same PGRs concentrations as for Shewa Robit. Meanwhile, the lowest percentage (39.2-40.8%) of shoot regenerations were recorded for all accession when the callus was cultured on media supplemented with 0.25mg/l TDZ (Table 12a). However, MS supplemented with BAP alone and combination with IBA did not induce shoot regeneration from callus culture. Calli that were cultured on this medium (BAP supplemented) was proliferated large quantities of healthy, green callus, but failed to differentiate shoots on all accessions type (Fig. 7B). Even on subculturing to respective media it continued to form massive callus rather than formation of organogenesis. And also, on the control medium the shoot regeneration was not noticed. However, the addition of TDZ to the medium improved the shoot induction potential of the calli as many shoot primordia were observed after 45 days of culture (Table 12a; Fig. 7C). Similar results were verified by Khurana-Kaul *et al.* (2010) who also showed that the combination of TDZ and IBA was more effective than the combination of BAP and IBA in Jatropha shoot regeneration using leaf segments as explants. Aishwariya *et al.* (2015) also reported that Thidiazuron (TDZ) is among the most active cytokinin like substances and it induces greater *in vitro* shoot proliferation than many other cytokinins in many plant species. Variation in the activity of different cytokinins can be explained by their differential uptake rate in different genomes, translocation rates to meristematic regions and metabolic processes

in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds also reported (Kaminek, 1992; Kumar *et al*., 2011).

4.3.2.2. Number of Adventitious Shoots per Callus

The mean number of adventitious shoots per callus was highly significantly ($P < 0.01$) affected by the main effects of PGRs, accessions and the interaction effect of the two factors (Appendix Table 7).

The highest adventitious shoot number (11shoot) was recorded for Shewa Robit callus cultured on MS media supplemented with 0.5mg/l TDZ and 0.1mg/l IBA followed by (10.4) and (9 shoot) shootlet per callus for Adami Tulu and Metema accessions, respectively. Whereas, the lowest shoot number (3.8-4) was recorded for all accessions, when the callus subcultured on MS media supplemented with 0.25mg/l TDZ (Table 12b). In this study, the number of shoots per callus was increased as the concentration of TDZ was increased from 0.25 to 1mg/l. These results suggest that TDZ plays a very important role in the formation of adventitious shoot buds of Jatropha, and these effects may be involved in stimulating *de novo* synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan, as well as increase in contents of endogenous cytokinin (Murthy *et al*., 1995; Murthy and Saxena, 1998). Besides, the ability of TDZ to induce high shoot regeneration efficiency in plant tissue has been reported for a number of species (Feyissa *et al*., 2005; Landi and Mezzetti, 2006).

Table 11. Effect of different concentrations of either BAP or TDZ in combination with IBA on percentage and number of shoot regenerated from leaf derived calli of Jatropha accessions after 45 days of culture growth. *Different letters (within rows and columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).*

a. Interaction effects of either BAP or TDZ in combination with IBA on percentage of shoot regeneration from callus derived from leaf of three Jatropha accessions.

PGRs conc. (mg/l)			**Adventitious shoot induction (%)**		
BAP	**TDZ**	**IBA**	**Metema**	**Adami Tulu**	**Shewa Robit**
0	**0**	**0**	-	-	-
0.5		**0**	-	-	-
0.5		**0.1**	-	-	-
0.5		**0.2**	-	-	-
1.0		**0**	-	-	-
1.0		**0.1**	-	-	-
1.0		**0.2**	-	-	-
	0.25	**0**	40.00 ±0.54^{jk}	39.20 ±0.49^{k}	40.80 ±0.34^{j}
	0.25	**0.1**	46.38 ±0.22^{h}	44.60 ±0.24^{i}	46.60 ±0.00^{h}
	0.25	**0.2**	50.00 ±0.00^{fg}	47.20 ±0.49^{h}	51.20 ±0.49^{f}
	0.5	**0**	43.36 ±0.16^{i}	43.20 ±0.00^{h}	43.52 ±0.19^{i}
	0.5	**0.1**	64.00 ±1.63^{b}	61.33 ±1.33^{c}	66.67 ±0.00^{a}
	0.5	**0.2**	53.30 ±1.35^{e}	54.40 ±1.1^{de}	55.50 ±0.16^{d}
	1.0	**0**	48.8 ±0.00^{g}	48.80 ±0.00^{g}	49.04 ±0.24^{g}
	1.0	**0.1**	31.20 ±0.80^{lm}	29.90 ±1.35^{mn}	32.40 ±0.40^{l}
	1.0	**0.2**	28.20 ±0.92^{o}	27.80 ±0.80^{o}	28.80 ±0.49^{no}
CV (%)			**4.63**		
LSD (5%)			**1.45**		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples, Conc. =concentration, '-' indicates no response

b. Interaction effects of either BAP or TDZ in combination with IBA on number of adventitious shoots regenerated from callus derived from leaf of three Jatropha accessions.

PGRs conc. (mg/l)			No of adventitious shoot per callus		
BAP	**TDZ**	**IBA**	**Metema**	**Adami Tulu**	**Shewa Robit**
0	**0**	**0**	-	-	-
0.5		**0**	-	-	-
0.5		**0.1**	-	-	-
0.5		**0.2**	-	-	-
1.0		**0**	-	-	-
1.0		**0.1**	-	-	-
1.0		**0.2**	-	-	-
	0.25	**0**	3.80 ±0.49^{o}	4.00 ±0.32^{o}	4.00 ±0.00^{o}
	0.25	**0.1**	5.00 ±0.00^{n}	5.00 ±0.00^{n}	5.00 ±0.00^{n}
	0.25	**0.2**	7.20 ±0.20^{gh}	7.40 ±0.24^{fg}	7.60 ±0.51^{efg}
	0.5	**0**	5.80 ±0.20^{klm}	6.00 ±0.32^{jk}	6.00 ±0.00^{jk}
	0.5	**0.1**	9.00 ±0.00^{c}	10.40 ±0.32^{b}	11.00 ±0.00^{a}
	0.5	**0.2**	7.80 ±0.20^{ef}	8.00 ±0.00^{de}	8.40 ±0.24^{d}
	1.0	**0**	6.40 ±0.24^{ij}	6.60 ±0.40^{i}	6.80 ±0.37^{hi}
	1.0	**0.1**	6.00 ±0.32^{jk}	5.96 ±0.13^{jkl}	6.06 ±0.0.06^{jk}
	1.0	**0.2**	5.42 ±0.05^{mn}	5.50 ±0.00^{lm}	5.60 ±0.10^{klm}
CV (%)			**10.77**		
LSD (5%)			**0.49**		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples, Conc. =concentration, '-' indicates no response

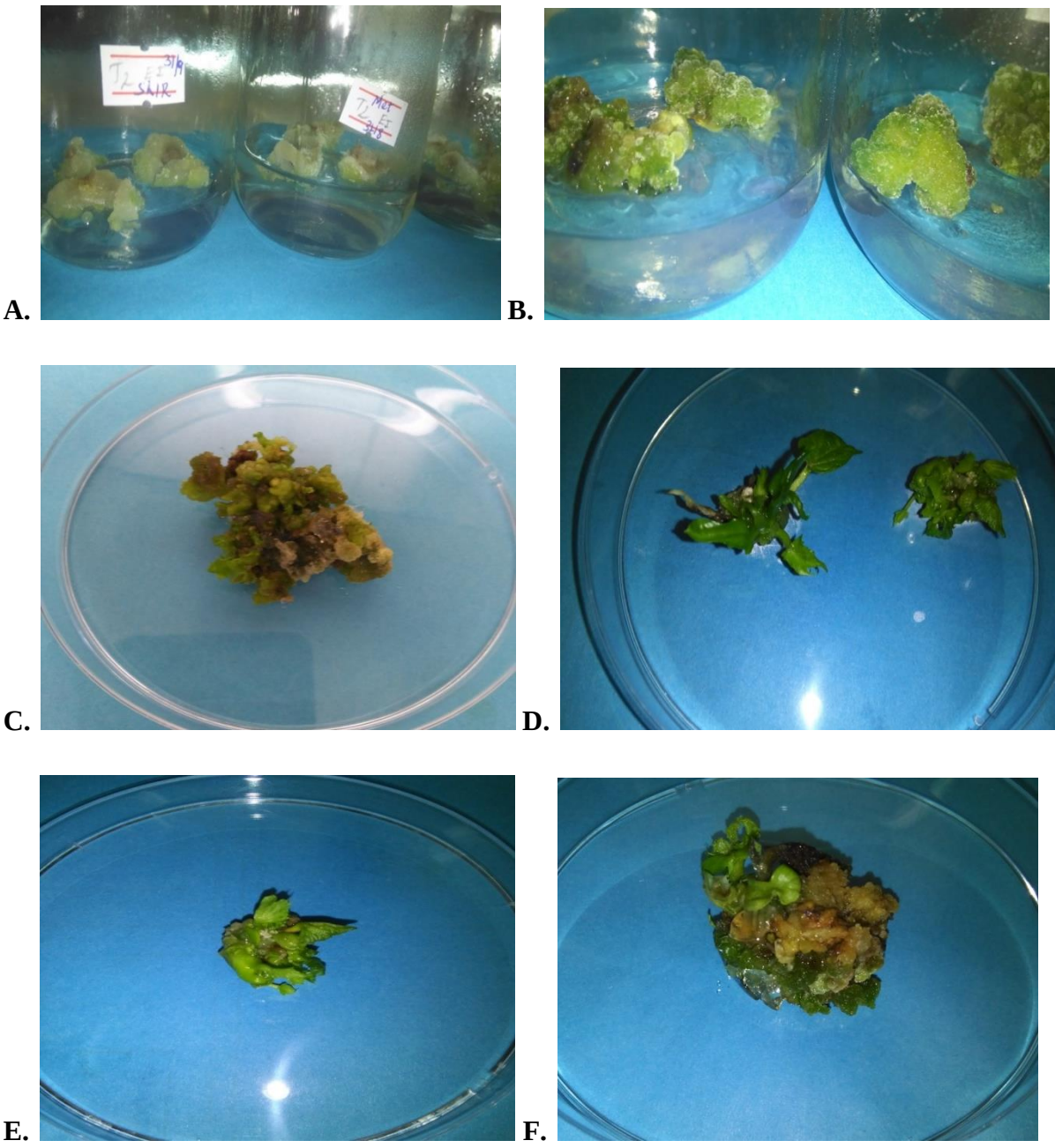

Figure 7. Multiple shoots regeneration from leaf derived callus of different Jatropha accession cultured at different concentration and combinations of either BAP or TDZ in combination with IBA after six weeks of culture. A) Leaf derived callus cultured for shoot regeneration; B) Leaf derived callus cultured on MS medium supplemented with 0.5m/l BAP + 0.1mg/l IBA; C) Adventitious shoot formation of Shewa Robit accession from leaf derived callus cultured at 0.5mg/l TDZ; D) Shoot regenerated from Shewa Robit leaf derived callus cultured on MS medium supplemented with 0.5mg/l TDZ and 0.1mg/l IBA; E) Shoot regenerated from leaf

derived callus of Metema accession cultured on MS medium supplemented with 0.5mg/l TDZ and 0.1mg/l IBA; F) Shoot regenerated from leaf derived callus of Adami Tulu accession cultured on MS medium containing 0.5mg/l TDZ + 0.1mg/l IBA.

4.3.3. Multiplication of Shoot Derived from Callus

4.3.3.1. Number of Shoot per Explant

The number of shoots proliferated per explants were significantly ($p<0.01$) influenced by PGRs combinations and concentrations, accessions as well as also by interaction effects of the two factors (Appendix Table 8).

The highest shoot number (3.5 shoots) was recorded for Shewa Robit on MS media supplemented with 0.5mg/l BAP whereas the highest shoot number (3.3 shoots) for both Metema and Adami Tulu were recorded on MS media supplemented with combination of 0.5mg/l BAP and 0.5mg/l Kn. The lowest shoot number (1.32-1.52) was recorded for all accession shoots cultured on hormone free MS media (Table 13). There are no conclusive reports on effect of growth regulators on number of shoot per shoot induced from calli for Jatropha but reports on other crops like sweet potato (Tasew, 2011) showed that the highest (2.4 shoots) number of shoots produced per shoots regenerated from leaf calli which cultured on MS media supplemented with 1mg/l BAP. The result of the present study is partially agreed with this study. In similar pattern, the number of shoots formed in both direct organogenesis (in experiment-I) and indirect organogenesis (in experiment-II) affected by variation in concentrations of different cytokinins (BAP and Kn). It is well established that, cytokinins (BAP and Kn) stimulate protein synthesis and participate in cell cycle control and if added into shoot culture media,stimulate lateral bud growth and thus causing multiple shoot formation by breaking shoot apical dominance (George*et al.*,2008). However, the number of shoot obtained through direct organogenesis (6 shoot per explant) is almost twice in rate as compared with indirect regeneration (3.5 shoot per explant). This shows that direct organogenesis is more efficient and easier to multiply shoots. In line with present study, Adiyecha *et al.* (2013) also reported that number of shoots formed were higher (10.3 shoots) in plantlets developed through direct organogenesis as compared to indirect organogenesis (8.1 shoots) via calli in *Curculigo orchioides.*

4.3.3.2. Number of Leaf per Shoot

Number of leaf per shootlet was also highly significantly affected ($P<0.01$) by the main effects of PGRs combinations and concentrations; however the main effect of accessions and the interaction effect of the PGRs and accessions was non-significant(Appendix Table 8).

The maximum mean number of leaf (4.47) per shootlet was recorded on MS media supplemented with 0.5mg/l of BAP followed by (4 leaves) per shootlet on media supplemented with combination of 0.5mg/l of BAP and 0.5mg/l of Kn (Table 14). Whereas, the lowest mean number of leaf (2 leaves per shoot) was recorded for shoots induced on plant growth regulator free MS media. The result showed that number of leaf formed tended to decrease as an increase in concentrations of BAP in combination with Kn. So far there is no report on effect of growth regulators on number of leaf per callus derived shoot for Jatropha but in the present study unlike to the *in vitro* shoots derived via direct regeneration, the number of leaf per shoot obtained is low. This might be due to different regeneration method that we used and it takes longer time to establish organ growth and development for the cells itself. However, Adiyecha *et al.* (2013) reported that shoots regenerated through callus produce large number of leaves (3.75) than shoots regenerated directly in *Curculigo orchioides.*

4.3.3.3. Shoot Length

The ANOVA revealed that there is significant difference on length of shootlet per explant due to the main effects of PGRs combinations and concentrations, accessions and interaction effect of the two factors (Appendix Table 8).

The maximum shoot length was recorded for Shewa Robit (2.26cm) on media supplemented with combination 0.5 of mg/l BAP and 0.5mg/l Kn followed by 2.24cm and 2.1cm for Adami Tulu and Metema accession, respectively, on the same PGRs concentrations. Meanwhile, the lowest shoot length (0.8-0.84cm) was recorded for all the three accession shoots developed on media supplemented with higher concentration of BAP (1.5 mg/l) (Table 13). In this experiment, maximum shoot length for all the three accessions was recorded on the media containing both BAP and Kn. This may be due to synergetic effects of BAP to Kn. However, the shoots produced showed stunted growth when the concentration of BAP increases for all

accessions. These results are in line with those of Sujatha *et al.* (2005) and Imtiaz *et al.* (2014) who found that the length of the shoot decreased with increasing concentration of BAP. This is mainly due to the fact that when the concentrations are beyond optimum level absorbed and used by cultured explants, which causes accumulation in the tissues and lead to toxicity to the plant. This most possibly leads to ceasing cell growth and sometimes cell death (Deore and Johnson, 2008; Imtiaz *et al.*, 2014). When indirect organogenesis and direct organogenesis is compared, the lower shoot length (2.26cm) was obtained from indirect organogenesis than direct organogenesis (3.2 cm). Likewise, Adiyecha *et al.* (2013) also found that shoot length was higher (9.3cm) in plantlets developed from direct organogenesis than indirect organogenesis which is 7.03cm in *Curculigo orchioides.*

Table 12. Effect of different concentrations of cytokinins on multiplication of shoot from callus of three Jatropha accessions after 30 days of culturing. *Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).*

Jatropha Accession	Cytokinin (mg/l)		No of shoot per explant	Length of shootlets (cm)
	BAP	Kn		
Metema	**0**	**0**	1.48 ±0.16^{op}	1.02 ±0.02^{q}
	0	**0.5**	2.36 ±0.16^{jkl}	1.39 ±0.02^{jkl}
	0	**1.0**	2.70 ±0.08^{fgh}	1.60 ±0.03^{g}
	0.5	**0**	3.10 ±0.10^{cd}	1.76 ±0.04^{ef}
	0.5	**0.5**	3.30 ±0.03^{b}	2.10 ±0.00^{b}
	0.5	**1.0**	2.76 ±0.05^{efgh}	1.90 ±0.00^{c}
	1.0	**0**	3.24 ±0.06^{bc}	1.26 ±0.02^{nop}
	1.0	**0.5**	2.86 ±0.02^{efg}	1.48 ±0.04^{hi}
	1.0	**1.0**	2.14 ±0.04^{mn}	1.24 ±0.02^{op}
	1.5	**0**	2.44 ±0.02^{ijk}	0.80 ±0.03^{r}
	1.5	**0.5**	2.24 ±0.04^{lm}	1.34 ±0.04^{klmn}
	1.5	**1.0**	2.00 ±0.00^{n}	1.20 ±0.00^{p}
Adami Tulu	**0**	**0**	1.32 ±0.08^{p}	1.10 ±0.03^{q}
	0	**0.5**	2.40 ±0.10^{jkl}	1.40 ±0.00^{ijkl}
	0	**1.0**	2.68 ±0.02^{gh}	1.58 ±0.02^{g}
	0.5	**0**	3.10 ±0.10^{cd}	1.80 ±0.00^{de}
	0.5	**0.5**	3.30 ±0.00^{b}	2.24 ±0.02^{a}
	0.5	**1.0**	2.76 ±0.08^{efgh}	2.04 ±0.06^{b}
	1.0	**0**	3.14 ±0.04^{bc}	1.28 ±0.05^{mnop}
	1.0	**0.5**	2.88 ±0.02^{ef}	1.46 ±0.02^{hij}
	1.0	**1.0**	2.12 ±0.05^{mn}	1.24 ±0.04^{op}
	1.5	**0**	2.40 ±0.00^{jkl}	0.82 ±0.02^{r}
	1.5	**0.5**	2.26 ±0.04^{klm}	1.32 ±0.02^{lmno}
	1.5	**1.0**	2.00 ±0.00^{n}	1.24 ±0.04^{op}
Shewa Robit	**0**	**0**	1.52 ±0.12^{o}	1.20 ±0.00^{p}
	0	**0.5**	2.60 ±0.06^{hi}	1.42 ±0.02^{ijk}
	0	**1.0**	2.70 ±0.00^{fgh}	1.70 ±0.00^{f}
	0.5	**0**	3.50 ±0.08^{a}	1.86 ±0.02^{cd}
	0.5	**0.5**	3.12 ±0.07^{bcd}	2.26 ±0.02^{a}
	0.5	**1.0**	2.80 ±0.05^{efg}	2.08 ±0.02^{b}
	1.0	**0**	3.26 ±0.04^{bc}	1.32 ±0.02^{lmno}
	1.0	**0.5**	2.94 ±0.02^{de}	1.52 ±0.05^{gh}
	1.0	**1.0**	2.22 ±0.02^{lm}	1.22 ±0.04^{p}
	1.5	**0**	2.48 ±0.02^{ij}	0.84 ±0.02^{r}
	1.5	**0.5**	2.28 ±0.02^{klm}	1.36 ±0.04^{klm}
	1.5	**1.0**	2.00 ±0.00^{n}	1.22 ±0.02^{p}
CV (%)			**5.72**	**4.58**
LSD (5%)			**0.18**	**0.083**

CV= Coefficient of variation, LSD=Least Significant Difference, SE=Standard Error

Table 13. Main effects of BAP and Kn in combinations and independently on the number of leaf per shootlet recorded 30 days after culture growth. *Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15).*

PGR concentration (mg/l)		**No of leaf per shootlet**
BAP	**Kn**	
0	**0**	2.00±0.05^{j}
0	**0.5**	3.00±0.00^{g}
0	**1.0**	3.33±0.02^{ef}
0.5	**0**	4.47±0.13^{a}
0.5	**0.5**	4.01±0.01^{b}
0.5	**1.0**	3.63±0.10^{cd}
1.0	**0**	3.43±0.13^{de}
1.0	**0.5**	3.80±0.07^{bc}
1.0	**1.0**	3.13±0.06^{fg}
1.5	**0**	2.43±0.05^{i}
1.5	**0.5**	2.70±0.07^{h}
1.5	**1.0**	2.17±0.06^{j}
LSD (5%)		**0.21**
CV (%)		**9.53**

CV= Coefficient of variation, LSD= Least Significant Difference, SE=Standard Error, n=number of samples

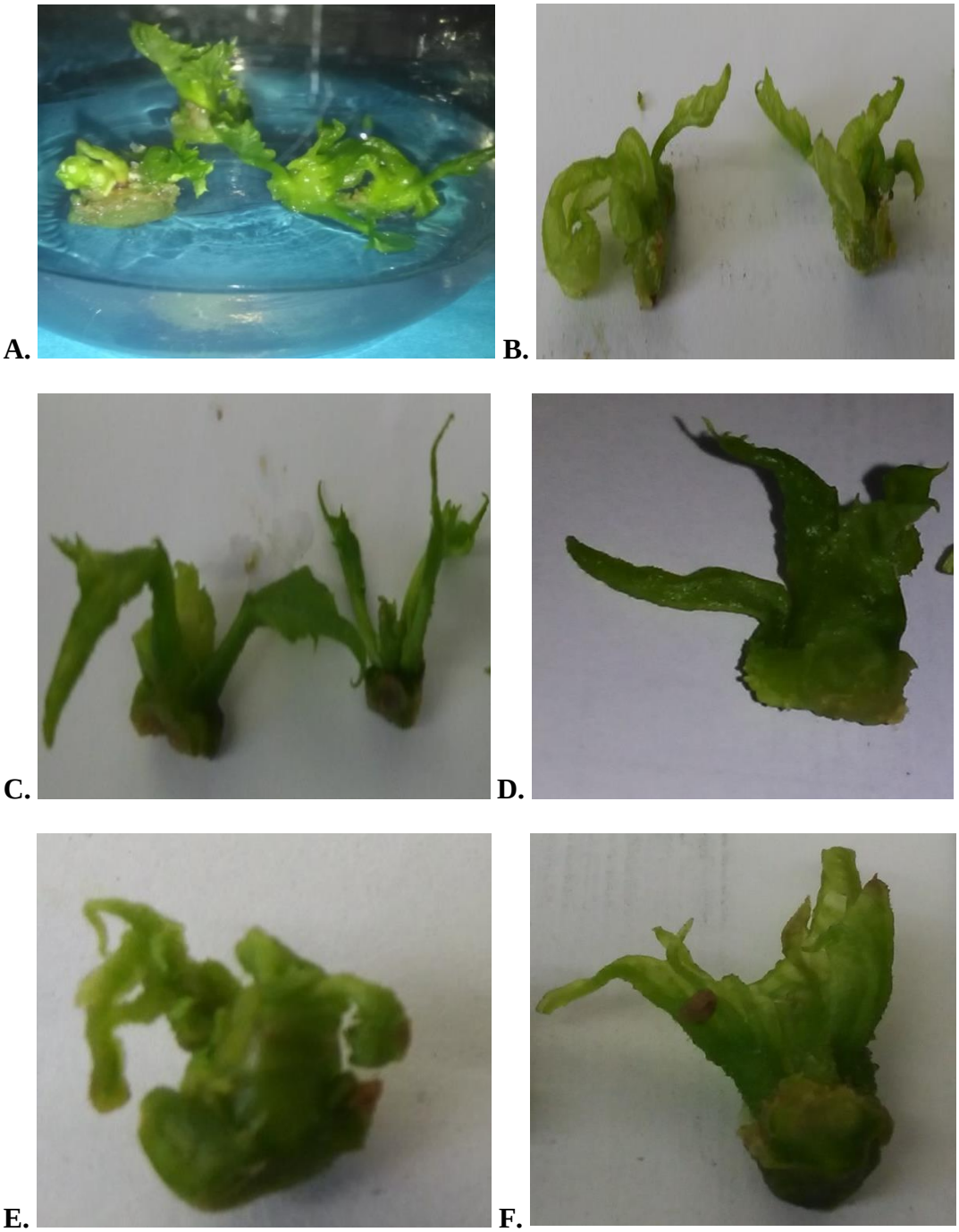

Figure 8. Multiplication and rooting of shoots derived from callus after four weeks of culture. A) Multiplied shoots regenerated from callus-derived shoots; (B, C and D) shows shoots of Shewa Robit, Metema and Adami Tulu regenerated on multiplication MS medium supplemented with 0.5mg/l BAP + 0.5mg/l Kn, respectively. E and F; indicate shoots showed moderate hyperhydricity on rooting medium.

4.3.4. Induction of Root for Callus-Derived Shoot

Elongated shoots of at least 1.5cm were also cultured on media containing various concentrations of IBA and NAA to induce adventitious root formation. However, unlike the *in vitro* shoots derived via direct organogenesis experiments; no adventitious roots were observed on any of the medium after four weeks.

Many shoots showed moderate hyperhydricity as indicated by callus formation at the base of shootlet and necrosis of the cut ends was observed (Fig 8; E&F). As alternative we also tried another protocol such as adding of Activated charcoal, since as per literature shootlet is expected to form adventitious roots on MS medium supplemented with various concentrations of IBA and 0.2% activated charcoal (Rajore and Batra, 20005). Activated charcoal is a common additive used in plant tissue culture primarily for its adsorbent properties scavenging phenols, reactive oxygen species and toxic accumulations from the PGRs (Thomas, 2008). These properties have led to improvements in shoot multiplication, elongation and rooting along with many other positive effects for *in vitro* growth (Thomas, 2008). Nonetheless, in the present study none of these treatments enable the shoots to form roots (data not shown) and however, only occurrence of unwanted callus was reduced. In general, this might be due to the longer period of time of the cultured on cytokinins especially TDZ and BAP could be inhibit root formation. This is in agreement with findings by Naik *et al*. (2000) and Mohamed *et al*. (2006), who have shown that long exposure of the shoots to high concentration of either TDZ or BAP, suppressed the capacity of shoots to form adventitious roots. In addition, it has been recorded that extra TDZ in medium might have the effectiveness of suppression of the growth of roots of Jatropha (Liu *et al*., 2015). Mundhara and Rashid (2006) has attributed triple role to TDZ in the cultures of Linum, where it was involved in the inhibition of roots formation, elongation, shortening and swelling of hypocotyls and tightening of cotyledons towards apex. Besides, the authors claim that the carryover of the TDZ to next stage of micropropagation is high and it is seen to cause the typical growth mishaps.

4.4. Acclimatization

In vitro induced shoots are very delicate and prone to sudden environmental changes that may damage the plantlets unless it is gradually adapted to the new environment. Thus acclimatization is essential to enable the rooted plantlets to adapt the natural environment in *ex vitro* conditions at temperature of 25±2^{o}c and 60-75% of relative humidity at greenhouse conditions. Therefore, plantlets from direct organogenesis experiments were transferred to greenhouse compartments at Holeta Agricultural Research Center for acclimatization.

Accordingly, 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 respectively after 30 days of acclimatization (Fig. 9B). 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 might result in excessive 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.

Table 14. Survival rate of plantlets derived from *in vitro* regeneration through direct organogenesis of three Jatropha accessions during acclimatization.

Accessions	**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

No. = number and % = percent.

A. 

B. 

Figure 9. Acclimatized plantlets of the three Jatropha accessions in the greenhouse. A) Plantlet covered by plastic bags for the first 15 days; B) After 30 days of growth in the greenhouse.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Jatropha (*Jatropha curcas* L.) is a drought resistant multipurpose perennial plant. Due to its enormous potentials of substituting oil fossil fuels and saving ecological system from further deterioration, its production and propagation has paramount importance. However, conventional propagation methods are not adequate enough for efficient production of this crop. Only the application of plant tissue culture and transformation techniques can expedite this process. The prerequisite for this is the establishment of efficient regeneration protocols. However, *in vitro* propagation study of Jatropha crop in Ethiopia is not yet studied. Therefore this research was designed to develop protocol for *in vitro* mass propagation of Jatropha accession namely; Metema, Adami Tulu and Shewa Robit accession through direct and indirect organogenesis. Based on the findings of the present investigation, the following conclusions are drawn:

- Surface sterilization with 2.5% and 3% of Berekina for 15 minutes exposure time is optimal for Jatropha leaf and nodal explants decontamination, respectively. Surface sterilization with Berekina gives not only the best result but also a safer option for both researchers and the environment and cost effectiveness compared to other disinfectants reported so far.

- For direct organogenesis, best shoot induction from nodal explant was obtained from MS medium containing 1mg/l BAP and 0.5mg/l IBA for all the three accessions. MS media supplemented with combination of 0.5mg/l BAP and 0.5 Kn mg/l followed by 0.5mg/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 0.25mg/l IBA followed by 0.5mg/l IBA with 0.25mg/l NAA were best for all root induction and growth parameters for the three accessions.

- *In vitro* propagation of Jatropha via indirect organogenesis was partially achieved through regeneration of adventitious shoot from leaf-derived callus tissue. The highest percentage of callus induction (100%) for all the three accessions were observed on MS media supplemented with combination of 1mg/l BAP and 1mg/l 2,4-D. The highest percentage of shoot regeneration (66.67%) was obtained from Shewa Robit accession on medium supplemented with combination of 0.5mg/l TDZ and 0.1mg/l IBA followed by 64% and 61.33% for Metema and Adami Tulu accessions, respectively, on the same PGRs concentration as for Shewa Robit. On the other hand, callus cultured on BAP alone and combined with IBA failed to regenerate shoots. *In vitro*-derived meristemoids shoots were multiplied on medium containing various concentrations of BAP and Kn to generate large quantities of plant material. Among the growth regulator combinations tested 0.5mg/l BAP followed by 0.5mg/l BAP in combination with 0.5mg/l Kn were best for all shoot induction and growth parameters for the three accession. Regenerated shoots were transferred to medium containing GA3 to elongate. However, the elongated shootlet which transferred into medium containing various concentrations of IBA and NAA failed to induce root growth.

- During acclimatization, plantlets from direct organogenesis experiments were transferred to greenhouse environment and maximum percentage of survival (86.67%) was obtained from shoots regenerated from Shewa Robit accession.

- Generally, in this study an efficient direct organogenesis protocol was developed for large-scale multiplication, propagation and conservation of Jatropha. This technique can be used for subsequent mass propagation of true-to-type plants and genetic transformation of the substantial biofuel plant. Besides, this study also gives useful insight of the protocols for the indirect regeneration of the Jatropha.

5.2. Recommendations

Future perspectives, based on the present study, should focus on the following areas:

- Different strength of MS media and liquid media should be tested for callus induction and shoot regeneration.
- Evaluation of variation in callus and regenerants using cytological analysis or molecular markers should be investigated.
- Subculturing should be done to examine the appropriate subculture stages at which maximum shoot multiplication takes place and the stage when the multiplication rate starts to decline. Also the genetic stability should also be assessed using molecular markers whether repeated sub-culturing stage affected stability of cultures or not.
- Further research for elongation and rooting induction protocol for indirectly regenerate shoots should be studied.
- Rooting response of multiplied shoots at each sub-culturing stage should be investigated.
- Alternate tissue culture protocols such as somatic embryogenesis should be studied; it may provide additional options for the *in vitro* regeneration of Jatropha.
- Further studies should focus on the performance of tissue culture generated plantlets in field condition for growth and yield related parameters to arrive at final conclusion of tissue culture efficiency in producing healthy planting materials of Jatropha.

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7. APPENDICES

Appendix Table 1. Nutrient composition and concentration of full strength MS medium

Stock Solution	**Nutrient(Salts/Vitamins)**	**Stock Solution(g/L)**	**Volume of stock for 1L medium (ml/L)**
Stock 1	NH_4NO_3	33.0	50
	KNO_3	38.0	
Stock 2	$MgSO_4.7H_2O$	18.07	10
	$MnSO_4.H_2O$	1.69	
	$ZnSO_4.7H_2O$	0.86	
	$CuSO_4.5H_2O$	0.0025	
Stock 3	$CaCl_2.2H_2O$	33.22	10
	KI	0.083	
	$CoCl_2.6H_2O$	0.0025	
Stock 4	KH_2PO_4	17.0	10
	H_3BO_3	0.62	
	$Na_2MoO_4.2H_2O$	0.025	
Stock 5	$FeSO_4.7H_2O$	2.78	10
	$Na_2EDTA.2H_2O$	3.726	
Stock 6	Myo-inositol	10.0	10
	Nicotinic acid	0.05	
	Thiamine-HCl	0.01	
	Pyridoxine-HCl	0.05	
	Glycine	0.2	

Others: Sucrose (30gm/l), Gerlite (2.5gm/l) and Ascorbic acid (10mg/l) are used

Appendix Table 2. ANOVA summary for the effect of duration of surface sterilization with Berekina on survival level of cultured explants (leaf and nodal explant)

		Mean Square
Source of Variation	DF	Survival Percentage
Expt	1	230.11**
Ber	3	2761.08**
Time	3	3140.17**
Expt *Ber	3	731.57**
Expt*Time	3	408.91**
Ber *Time	9	1136.06**
Expt * Ber *Time	9	280.70**
Error	64	6.85
CV (%)		5.31

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, CV= coefficient of variation, Expt=Jatropha explants and Ber= Berekina (local bleach).

Appendix Table 3. Mean square estimates of *in vitro* shoot formation and growth parameters of the three Jatropha accessions as affected by PGRs (BAP and IBA) combination.

		Mean Square	
Source of Variation	DF	Percentage of Shoot formation	Days to shoot induction.
PGR	11	14645.65**	503.29**
Accession	2	3.71ns	5.83ns
PGR*Accession	22	6.89**	0.34ns
Error	144	2.25	0.21
CV (%)		2.92	4.11

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, PGR= Plant Growth regulator and CV= coefficient of variation

Appendix Table 4. Mean square estimates of ANOVA for *in vitro* shoot multiplication and growth parameters of the three Jatropha accessions as affected by PGRs (BAP and Kn) combination.

		Mean Square		
Source of Variation	DF	Number of shoots	Number of leaf	Shoot length
PGR	11	20.45**	26.23**	2.770**
Accession	2	0.84**	0.12ns	0.026**
PGR*Accession	22	0.10*	0.02ns	0.004*
Error	144	0.05	0.13	0.002
CV (%)		6.98	7.93	2.03

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, PGR= Plant Growth regulator and CV= coefficient of variation

Appendix Table 5. Mean square estimates of *in vitro* root induction and growth parameters of the three Jatropha accessions as affected by PGRs (IBA and NAA) combination.

		Mean Square			
Source of Variation	DF	Days for root induction.	Rooting percentage	Number of roots	Root length
PGR	11	747.88**	12901.41**	45.62**	24.05**
Accession	2	0.14ns	4.86**	0.08ns	0.24**
PGR*Accession	22	0.04ns	3.69**	0.11ns	0.05*
Error	144	0.17	1.09	0.12	0.03
CV (%)		2.82	1.89	12.28	7.63

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, PGR= Plant Growth regulator and CV= coefficient of variation

Appendix Table 6. Mean square estimates of ANOVA for *in vitro* Callus induction and growth parameters of the three Jatropha accessions as affected by PGRs (BAP &2,4-D) combination.

		Mean Square		
Source of Variation	DF	Days to callus formation	Percentage of callus formation	Callus fresh weight
PGR	15	836.48**	18707.79**	9.1006**
Accession	2	0.20ns	3.41ns	0.0012ns
PGR*Accession	30	0.11ns	2.05*	0.0003ns
Error	192	0.17	1.32	0.0004
CV (%)		3.32	2.19	1.75

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, PGR= Plant Growth regulator and CV= coefficient of variation

Appendix Table 7. Mean square estimates of *in vitro* adventitious shoot formation and growth parameters of the three Jatropha accessions as affected by cytokinins (TDZ &BAP) and Auxin (IBA).

		Mean Square	
Source of Variation	DF	Percentage of shoot regeneration	Number of adventitious shoots
PGR	15	8973.49**	193.67**
Accession	2	25.58**	1.29**
PGR*Accession	30	3.70**	0.33**
Error	192	1.37	0.15
CV (%)		4.63	10.77

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, PGR= Plant Growth regulator and CV= coefficient of variation

Appendix Table 8. Mean square estimates of ANOVA for *in vitro* callus-derived shoot multiplication and growth parameters of the three Jatropha accessions as affected by PGRs (BAP &Kn) combination.

		Mean Square		
Source of Variation	DF	Number of shoots	Number of leaf	Shoot length
PGR	11	4.58**	8.546**	2.299**
Accession	2	0.12**	0.207ns	0.086**
PGR*Accession	22	0.03*	0.009ns	0.008*
Error	144	0.02	0.091	0.004
CV (%)		5.72	9.53	4.58

*and**= significant at 5% and 1% probability levels respectively, ns=non-significant at 5%, DF=Degree of freedom, PGR= Plant Growth regulator and CV= coefficient of variation

BIOGRAPHICAL SKETCH

The author was born in April, 1994 from his father Fufa Gudeta and his mother Degatte Kelbessa at Wedessa Kebele, in Ambo District, West Shewa zone of Oromia regional state, Ethiopia. He attended his elementary school at Ambo from 2001-2008. After successfully passing his grade eight qualifying exam he joined Ambo secondary and preparatory school and attended his education from 2009 to 2012. After completion of his preparatory school education, he joined Madda Walabu University and obtained the Bachelor of Science degree in Plant Science in July 2015. Upon graduation, he was employed by Ministry of Education as Graduate Assistant at Jinka University. Then he joined the School of Graduate Studies at Hawassa University in October 2015 to pursue his study leading to M.Sc. degree in Horticulture.