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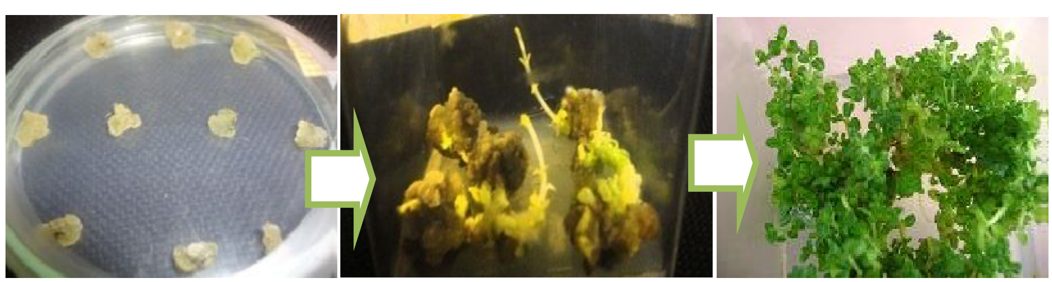
In Vitro Regeneration of *Plectranthus edulis* (Vatke) from leaf derived callus



A Thesis Submitted to the School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Nitsuh Aschale

Addis Ababa, Ethiopia

January, 2014

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Abstract

In vitro regeneration of *Plectranthus edulis* (Vatke) from leaf derived callus

Nitsuh Aschale

Addis Ababa University, 2014

Plectranthus edulis *(Vatke) Agnew (syn.* Coleus edulis *Vatke), is a diploid, dicotyledonous plant, belonging to the Labiateae (Lamiaceae) family. It is a major source of food in many parts of Ethiopia and also used as a traditional medicine to treat different diseases. This plant is vegetativelly propagated mainly by tubers. Vegetativelly propagated plants are mostly affected by different diseases. These diseases reduce the yield and results in poor quality crops. The establishment of* in vitro *plant regeneration system in* P. eduils *is of potential importance to quality improvement and genetic transformation. The study was conducted to develop* in vitro *regeneration protocol for* P. eduils*. For this study, calli were induced from leaf explants excised from three-week-old shoots grown on MS medium containing different concentrations of NAA and BAP. The highest (100 %) callus induction was obtained on MS medium supplemented with 1.5 mg/l NAA+1.0 mg/l BAP and 2 mg/l NAA+0.5 mg/l BAP. Among the different growth regulators combinations used for multiplication, maximum (58.68±6.57) mean numbers of shoots were obtained on 0.5 mg/l BAP in combination with 0.4 mg/l* GA_3. In vitro *developed shoots were rooted best with mean root number of 10.15±0.95 on MS medium supplemented with 2.0 mg/l IBA. All* in vitro *rooted and 78 % of microshoots were survived in the glasshouse. This protocol can be used to improve this plant through* in vitro *screening and genetic transformation.*

Key words: callus induction, growth regulator, organogenesis, regeneration

Acknowledgments

First and foremost, I would like to thank my advisor, Dr. Tileye Feyissa for his continuous and unreserved support and critical reading of the thesis as well as providing the necessary resources unlimitedly during the course of this study.

I am grateful to acknowledge Addis Ababa University and Institute of Biotechnology for providing me all academic supports during this study.

I would like to express my gratefulness to my colleague Helen G/Medihen for financially supporting me and for her unreserved guidance during all activities done from the beginning to the end of my study. Without her support, I would not be in a position to go this far.

I would like to sincerely thank Wubante Mekonnen and Melaku Tilahun for their unreserved support and grammatically editing this paper, Teslim Yimam for helping me during statistical analysis of data.

I would also take this opportunity to thank my family, whose commitment to my studies was helpful. Thank you Aynadis Aschale and your husband Getachew Anidualem for all that you sacrificed and my father priest Aschale Yenesew and my mother, Adametu Engida for giving me your all rounded support and love.

Finally, I thank my fellow graduate students who supported me in all activities done from the beginning to the end of this study. They greatly helped me when I faced problems.

I may really forget wonderful people whose involvement was great, but I would like to say thank you.

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List of Abbreviations

ANOVA	Analysis of Variance
BAP	6-benzyl Amino Purine
EDTA	Ethylene Di-Amine Tetra Acetic Acid
EHNRI	Ethiopian Health and Nutrition Research Institute
FAO	Food and Agricultural Organization
GA_3	Gibberellic Acid
IAEA	International Atomic Energy Agency
IBA	Indole-3-Butyric Acid
IBC	Institute of Biodiversity Conservation
Kpa	kilo Pascal
LSD	Least Significant Difference
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
PGRC	Plant Genetic Resources Center
PGRs	Plant Growth Regulators
TDZ	Thidiazuron

1. Introduction

In many countries, tuber plants are used as major calorie contributors and have the highest rate of dry matter production per day. It is not only enriching the diet of the people but also possesses medicinal properties to cure many diseases. In addition to the above advantages, many tropical tuber crops contribute in the preparation of stimulants, tonics, carminatives and expectorants. It is also rich in dietary fiber and carotenoids (Edison *et al.*, 2006).

The genus *Plectranthus* belongs to the family *Labiatae (Lamiaceae*). It is a large genus containing about 300 species mostly found in Tropical Africa, Asia and Australia (Paton *et al*., 2004 cited in Lukhoba *et al*., 2006). Among 300 species of *Plectranthus*, 62 species are used as medicines, ornamentals, foods, flavors, etc. In addition to the above uses, *Plectrathus* species are also used as food plants by the larvae of some Lepidoptera species including the Engrailed (*Ectropies crepusularia*). Species with ethnobotanical uses are not randomly distributed throughout the genus but are related (Lukhoba *et al*., 2006).

Plectranthus edulis is one of the most important indigenous tuber crops in Ethiopia. It is a diploid, dicotyledonous plant and also occurs as wild. It can grow at the mid to high altitudes areas in the south, north and south-west of Ethiopia. Its height reaches up to 1.5 m and produces edible tubers on below-ground stolons. This growing habit of *P. eduils* resembles that of the Irish potato (Mulugeta Taye, 2008).

P. edulis has a long history of local usage and is important to the cultural, social and economic life of households (Abebe Demissie, 1988 cited in Yeshitila Mekbib and Webiull, 2012). It is traditionally recommended as a special food in the community for people who are recovering from illness, probably owing to its high digestibility. It is also reported that it has no impact on the stomach whatever amount is consumed (Yeshitila Mekbib and Webiull, 2012) and serves as medicinal plant, eating the boiled root can avoid loss of appetite (Moa Megersa, 2010).

For unknown reasons, farmers and the Ministry of Agriculture claim that the total tuber production has declined considerably over the last few decades. However, information on the growth and development of *P. edulis* and cultural practices of this tuber plant is scarce. Even basic knowledge on the crop is not available (Mulugeta Taye, 2008).

Vegetativelly propagated crops are mostly affected by diseases like virus, fungi, bacteria, insects and nematodes (Wharton and Kirk, 2007; IAEA, 2004). Thus diseases are problems that contribute significantly to yield loss in tubers crops among which *P. eduils* is the one. Application of modern biotechnologies in *P. eduils* would be decisive to improve the quality as well as quantity of this tuber by means of producing plants resistant to insect, herbicide, fungi, bacteria and nematode through *in vitro* screening and genetic engineering. These are performed by utilization (exploitation) of gene transfer by genetic transformation and somatic hybridization which requires the control of plant regeneration from tissue culture techniques. Then, development of a reliable *in vitro* plant regeneration procedure is a pre-requisite for its improvement by creating genetic variability via biotechnological methods involving direct gene transfer (Sihachakr *et al.*, 1997). Success of any transformation strategy depends largely upon the regeneration capability of the target explants (Chugh and Khurana, 2003).

Therefore, developing a regeneration protocol for *P.eduils* is a pre-requisite for introducing new genes and transformation protocol.

2 Literature Review

2.1 Taxonomy and Morphology of *Plectranthus edulis*

P. edulis (*Coleus edulis*) is a tuber crop that belongs to the family Labiatae (Lamiaceae). It is small hairy, succulent herb about a maximum of 150 cm high, with ovate and shallowly serrate leaves that are cultivated on small-scale basis (Yeshitila Mekbib, 2007).

P. edulis is highland crop that has different shapes, colour and size. Among the different species of *Plectranthus*, *P. edulis* (Vatke) Agnew (syn. *Coleus edulis* (Vatke), is a diploid, dicotyledonous plant, locally called Oromo potato (Oromo dinch), Wolaita dono (Wolaita dinich), Gurage Dinch, Agew Dincha and Ethiopian potato (Mulugeta Taye *et al.*, 2007; Yeshitila Mekbib, 2007). It is originated in Ethiopia (Dandena Gelmesa, 2010; IBC, 2005).

It is composed of the mother tuber piece, sprouts, main stems, branches, leaves, inflorescences, fruits, seeds, roots, stolon, and tubers. The stolon are very long and are initiated on buds of main stems and primary branches, first only on below-ground nodes, but later also on the lower aerial nodes. Tubers are produced upon swelling of the tip or middle part of stolons. Tubers that are formed from along the stolon are longer than the stolon tip. The tubers of *P. edulis* are stem tubers, like those of Irish potato. Hence, the colour of the vegetative parts, stolons and tubers varied depending on the cultivar (Mulugeta Taye *et al.*, 2012).

There are different local cultivars that have been identified by farmers (Mulugeta Taye *et al.*, 2007). Similarly, Yeshitila Mekbib (2007) reported that earlier farmers of Wolaita had been growing a wider diversity of local cultivars of *P. edulis* for various reasons. These days, however, they have specialized on few cultivars that they thought would meet their needs best. At least six local cultivars, namely, ‘Lofuwa’, ‘Unnuka’, ‘Chenkuwa’, ‘Chedia’, ‘Merchia’ and ‘Keytaria’, currently growing by farmers and all of them had been selected by them (Yeshitila Mekbib and Webiull, 2012).

Among those, the three local cultivars, namely ’Chenkuwa’, ’Lofuwa’ and ’Unnuka’ were found to be common and widely and evenly distributed in all the growing areas of Wolaita. The dominance of the three major local cultivars was found to be associated with the specific qualities attached to each cultivar (Yeshitila Mekbib and Webiull, 2012). Their differences are indicated below in Table 1.

Table 1 Descriptions of local Ethiopian potato (*Plectranthus edulis* (Vatke) Agnew) varieties by farmers in Sodo Zuria district, South Ethiopia

Characters	**Lofuwa**	**Unnuka**	**Chenkuwa**
Drought tolerant	Poor	Good	Very good
Maturity	Early	Intermediate	Late
Taste	Good	Intermediate	Very good
Disease resistance	Good	Good	Good
Tuber yield	Good	Good	Intermediate
Tuber size	Big	Intermediate	Small
Tuber colour	Cream	White	Red purple
Plant height	Tall	Short	Intermediate
Shelf life	Poor	Intermediate	Very good
Marketability	Seasonal	Seasonal	All times

Source: Ethiopian Health and Nutrition Research Institute (EHNRI, 1997).

2.2 Origins and growing condition

In terms of crop diversity, Ethiopia is one of the richest genetic resource centers in the world. This is principally attributed to the diverse farming systems, socio-economics, cultures and agro-ecologies. Crop plants such as coffee (*Coffea arabica*), safflower (*Carthamus tinctorius*), Tef (*Eragrostis tef*), noug (*Guizotia abyssinica*), Anchote (*Coccinia abyssinica*), Enset (*Ensete ventricosum*), *Plectranthus edulis* (Vatke) Agnew (syn. *Coleus edulis* Vatke) are known to have originated in Ethiopia (Dandena Gelmesa, 2010; IBC, 2005 and 2007). Enset, Irish potato, sweet potato, yam, cassava, taro and *Plectranthus edulis* are tuber crops, which are the major source of food in Southern Ethiopia (Andargachew Gedebo *et al*., 2011).

Consumption of *P. edulis* was related with the coming of the negroid people in 3000 BC, who penetrated the plateau from the west, bringing with them agriculture of the Sudanic type (sorghum, cowpea, yam, okra, etc.) (Murdock, 1959 cited in Mulugeta Taye, 2008).

It has been grown in mid and high altitude areas ranging from 1880 to 2200 meters above sea level (Abebe Demissie, 1988) in the North, South and West of Ethiopia and is primarily cultivated for its tubers (Mulugeta Taye, 2008). The genus *Plectranthus* occurs both as wild and cultivated species. Thirty two wild species are found in Ethiopia (Hedberg *et al.*, 2006). The wild species are found throughout the country (PGRC, 1995; IBC, 2007). These tubers are a good source of carbohydrates consumed after cooking (Mulugeta Taye *et al.*, 2007).

2.3 Significance and uses

P. edulis has been grown and used as a major source of food in many parts of Ethiopia and is a main source of carbohydrates for the farming community specially in Northern part (EHNRI, 1997). The tubers are rich in energy and have a slightly higher carbohydrate concentration after cooking than Irish potato (EHNRI, 1997; Mulugeta Taye, 2008). The leaf is cooked and eaten as vegetable in some western parts of Ethiopia, particularly in the Kefa area (Zemede Asfaw and Zerihun Woldu, 1997) and also used as a traditional medicine to cure different diseases.

Additionally, farmers highly appreciate *P. edulis* and indicate it satisfies one's hunger better than other tuber crops. They believe that this tuber is very important for it makes them energetic, and leads them to have more children (Mulugeta Taye, 2008).

P. edulis is one of the most preferred foods and often served to esteemed guests. It is traditionally recommended as a special food in the community for people who are recovering from illness, probably owing to its high digestibility. It is also reported that it has no impact on the stomach whatever amount is consumed (Yeshitila Mekbib and Webiull, 2012).

Yeshitila Mekbib (2007) indicated that *P. edulis* is eaten during Meskel Holiday, a popular religious holiday in Ethiopia, as one component of the diversified dishes prepared for celebration. The cultivations of *P. eduils* tubers largely depend on these popular religious holidays. This is because, as a tradition, people start to eat *P. edulis* shortly after the end of this religious holiday. In addition to the above advantage, the dried stems are also used as firewood whenever there is shortage of wood. It is one of the main important tuber plants to reduce the consumption of other wood for the purpose of fire.

According to Yeshitila Mekbib and Webiull (2012), most farmers reported that *P. edulis* contributes less to household food security as compared to other root crops such as *Solanum tuberosum* and *Ipomoea batatas*. This is mainly attributed to its short shelf life. Because of this reason people use *P. edulis* to fill a gap during times of food shortage.

P. edulis has sufficient amounts of micro and macro nutrients with relatively higher food energy when cooked than *S. tuberosum*, and the fat and calcium contents are higher than that of *S. tuberosum*. In addition to the above unique properties, *P. eduils* also contains protein as much as twice that of *Ipomoea batatas* when cooked. Therefore, the cooked tubers have more energy, fiber and carbohydrate compared to the raw tuber (Yeshitila Mekbib and Webiull, 2012). However, the latter is richer in nitrogen, protein, calcium, phosphorous, iron and niacin than the cooked ones (EHNRI, 1997).

Table 2 Nutritional content of *Plectranthus edulis, Solanum tuberosum* and *Ipomoea batatas* (all values per 100 g of edible portion)

Composition	*P. eduils*		*S. tuberosum*		*I. batatas*	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
Food energy (calories)	69.00	100.60	103.70	89.70	136.00	134.20
Moisture (%)	81.90	73.80	73.10	76.80	67.40	65.60
Nitrogen (g)	0.30	0.24	0.30	0.26	0.30	0.13
Protein (g)	1.50	1.00	1.30	1.10	1.30	0.50
Fat (g)	0.20	0.20	0.10	0.10	2.00	0.20
Carbohydrate (incl. fiber) (mg).	15.30	23.70	24.40	21.10	28.20	32.60
Fiber (g)	0.70	1.00	1.40	0.90	1.10	1.50
Ash (g)	1.10	1.30	1.10	0.90	1.10	1.10
Calcium (mg)	29.00	19.00	14.00	9.00	52.00	35.00
Phosphorous (mg)	90.00	62.00	57.00	49.00	34.00	54.00
Iron (mg)	9.30	1.10	2.30	1.50	3.40	0.90
Thiamin (mg)	-	0.11	0.08	0.05	0.08	0.06
Riboflavin (mg)	-	0.32	0.08	0.09	0.05	0.01
Niacin (mg).	0.70	0.30	1.00	0.80	0.90	0.40

Source: Yeshitila Mekbib, 2007

2.4 Harvesting and seed tuber storage

Depending on the type of cultivar, the crop is harvested 6-8 months after planting. The harvesting time stretches from September to November. Tubers were harvested by digging them up with a digging hole. It takes place gradually depending upon the need of the family. Bulk harvesting can be done but the crop is more often harvested as needed. The process involves completely pulling up or digging out the plants. The tubers are long, brittle and finger-like and are easily broken (Mulugeta Taye *et al.,* 2012).

No research has been published on the effect of different storage conditions on the seed tuber performance of *P.eduils* (Mulugeta Taye, 2008); farmers store their produce (whether for consumption, sale or seed) in the production beds. This is the only traditional storage technique used. However, farmers' traditional knowledge and practice on seed tuber storage techniques are used like to keep the tubers alive until the next planting seasons by covering the fields with available mulching materials to protect them from direct sunlight (Yeshitila Mekbib, 2007). Farmers also store in the ground, i.e. in the place where the crop was planted, for a maximum period of five months, but usually for a shorter time (Mulugeta Taye *et al*., 2007).

2.5 Market demand of *Plectranthus edulis* in Ethiopia

P. edulis is propagated vegetativelly by tubers, and the seed tuber for planting was obtained from individually selected tubers of the previous harvest or bought in the market. Market demand is another selection criterion to use this tuber crop. The farmers usually sell their produce during two peak seasons. These are from September to

November at harvest and during the planting time of March and April (Yeshitila Mekbib and Webiull, 2012).

Farmers reported that there is a direct relationship between palatability and shelf life. The early maturing cultivars often have larger tuber sizes but have relatively short shelf life. These cultivars can be consumed and sold only in the right physiological maturity (i.e. mostly in September). Otherwise the contents of the tubers will be changed to fiber. As a result, its market value will be low. Thus, farmers prioritize their local cultivars depending on their objective (i.e. for market or home consumption). Those local cultivars which can be consumed for a relatively long period of time without a significant reduction in their food qualities often get a better price regardless of season. As a matter of fact, it was common to see local cultivars with specific demands and price variation (Yeshitila Mekbib, 2007).

2.6 Vegetative propagation and management practices

P. edulis has seeds which are brown or black, ovoid-shaped and smaller than 1.0 mm. The fruit consisted of four seeds included in the persistent calyx (Mulugeta Taye *et al.,* 2012). But, it is propagated vegetativelly by using the edible parts, i.e. the tubers, by planting 2−3 tuber pieces of a broken seed tuber in one planting hole. Cultural practices include tipping (removal of the apical stem parts), earthing up (piling up of the soil around the stems) and manuring (Mulugeta Taye *et al.,* 2007) also used during planting.

The tuber pieces, which can be planted as sprouted or unsprouted are principally obtained from the previous crop or market. Most farmers said that the cultivation technique of *P. edulis* is laborious (Yeshitila Mekbib and Webuil, 2012). But, in general traditional methods are considered to be complex in nature and take comparatively a longer period to produce a variety with desired characters (Grafius and Douches, 2008).

2.7 Major problems associated with *P. edulis* production

Propagation from vegetative parts, grafting and sap can transmit viruses from one plant to another. Many varieties of vegetativelly propagated crops decline in performance with viral accumulation and must be discarded. Since the viruses do not produce visible symptoms, they cannot be destroyed by using plant protection chemicals. However, the presence of viruses in plants can reduce the yield and results in poor quality of crops (IAEA, 2004). The use of virus resistant varieties or eradicated disease free initial seed production material is preferred for avoiding crop yield or quality losses (Danci *et al.*, 2011).

In some areas, the most important economic damage to potato is caused by insects and nematodes, which loses quality and becomes susceptible to soil pathogens (Gregory, 1977). *P. edulis* plant is attacked by diseases and insect pests like other vegetativelly propagated tubers. A variety of methods now exist for crop improvement, among them genetic transformation is the one. Novel gene sequencing, cloning and insertion, as well as functional genomics, are tools that will benefit the improvement of economically important potato cvs. and dihaploid genotypes through reliable transformation protocols (Tican *et al*., 2007). Genetic transformation is an important tool in addressing increasing worldwide demands for crops with higher agricultural production and more nutritional value. For a genetic transformation system to be effective, it is not only necessary to have a suitable transformation mechanism to deliver the foreign DNA, but it is also essential to develop a rapid and efficient regeneration protocol (Chakravarty *et al.,* 2007). Because, establishment of an efficient *in vitro* regeneration protocol is a pre-requisite to facilitate successful plant genetic transformation (Soto *et al.,* 2007). Hence, it is better to develop an effective *in vitro* regeneration protocol for *P. eduils* for further work like other tuber plants.

2.8 Tissue culture

Plant Tissue Culture is the science of aseptic (free from any microorganisms) growing of any plant protoplast, cell, tissue and organ from mother plant on sterilized artificial growth media (Singh, 2000; George *et al.,* 2008). It produces elite plants for multiplication, disease free and stable plants with well-developed root system and their establishment and growing capacity are very high. It is also important for production of high quality planting material which is uniform, healthy, disease free and having accelerated growth (Neal, 2008).

It is classified into two; namely: cultures of unorganized tissues and cultures of organized tissues. Cultures of unorganized tissues are: callus cultures, cell suspension cultures, protoplast culture and microspore culture. In another cases, culture of shoot, root, embryo, nodal, leaf sheath, fruit and flower cultures are classified as cultures of organized tissues (George *et al*., 2008).

Tissue culture provides need of embryo culture (rescue) in which viable offspring is obtained from seeds with a tendency for embryo abortion or when a viable seeds are limited in number (Gamborg and Phillips, 1995). It offers several advantages over conventional propagation techniques.

It enables to produce millions of uniform and disease or pathogen free plants from a single explant and this has got a great advantage in realizing improved varieties within a short period of time for commercial as well as consumption purpose (Neal, 2008).

Plant tissue culture techniques are also central to ground-breaking areas of applied plant science, including plant biotechnology and agriculture. For example, selected plants can be cloned and cultured as suspended cells from which plant products can be harvested (Mineo, 1990).

2.9 Tissue culture of *P. edulis*

In earlier studies, only micropropagation of *P. edulis* has been reported (Mesfin Tsegaw, 2012 and Tesilm Yimam, 2013). However, both of them were only on mass-propagation of this tuber crop. Yet, *in vitro* plant regeneration from cells, tissues, and organ cultures is a fundamental process for the application of plant biotechnology to plant propagation, plant breeding and genetic improvement (Alejo, 2001).

Callogenesis and plant regeneration from cultured tissue explants are basic requirements for the application of tissues culture for genetic improvement. It can be regenerated from explants such as leaves, stems, petioles and tubers following an intermediate callus phase (Prematilake and Mendis, 1999).

Callus formation is central to many investigative and applied tissue culture procedures. It is a potential source to create genetic variability in micropropagated plants (Yasmin *et al.*, 2011). Additionally, callus can be multiplied and later used to clone numerous whole plants. Various genetic engineering protocols employ callus initiation procedures after

DNA has been inserted into cells; transgenic plants are then regenerated from transformed callus. In other protocols, callus is generated for use in biotechnological procedures such as the formation of suspension cultures from which valuable plant products can be harvested (Mineo, 1990).

In addition to the above advantages, today availability of this method for *in vitro* callus culture opens new possibilities for establishing a continuous cell culture system for easy and rapid isolation of bioactive compounds. Large scale suspension cultures, in a manner similar to that of microbial fermentation, will be suitable for industrial production of useful plant-derived photochemical commonly used as pharmaceutics, cosmetics and food additives (Sutan *et al.,* 2010). This is one of the decisive indications for other important plants in order to extract chemicals by using continuous subculture of callus.

On other cases, plants can be regenerated from protoplasts which have more variability than those directly regenerated from other explants (Jones, 1994). Accordingly, some authors have argued that plants regenerated from direct somatic embryogenesis and organogenesis ought to contain fewer mutations than those regenerated via callus phase (Gloriada *et al.,* 1999).

Hence, indirect organogenesis is often associated with somaclonal variation (Chen and Henny, 2006). But, plants regenerated through callus culture are genetically identical to the source materials free from pathogen and it is possible to produce a huge number of plantlets in a very short period of time. It is also a pre-requisite to facilitate successful plant genetic transformation (Soto *et al*., 2007) and identify factors that affect transformation (Tileye Feyissa *et al.*, 2007).

According to Gonzalez *et al*. (1999), leaves had a higher regeneration frequency compared to stem. Therefore, it considers that leaves are suitable target explants in transformation studies based on this organogenesis-mediated protocol. In addition to the above, *in vitro* regeneration is fundamental to protect the aseptic culture from infection and to prevent desiccation of the plant and the nutrient medium, because it is carried out in closed vessels, which unintentionally restrict the exchange of gases between the vessel atmosphere and the outside air (Joosten and Woltering, 1994).

Liu *et al*. (1993), states that genetic transformation offers great potential for improving the disease or pest resistance and nutrition quality of sweet potato (*Ipomoea batatas* (L) Lam.). Additionally, somaclonal variation in potato calli can be utilized to find suitable variants with desired characters, such as drought or salt stress tolerance (Ehsanpour *et al.*, 2006 cited in Munir *et al.*, 2011). It is an appropriate method to eliminate fungus and bacteria. It is also useful in genetic variation and selection for desired trials performed *in vitro* (Khatun *et al*., 2003).

In potato transformation, early identification of somaclonal variants can be beneficial in eliminating undesired variants that may affect morphological characters and agronomic functions (Munir *et al.*, 2011). Hence, an efficient regeneration protocol from the callus cultures is a pre-requisite for successful application of genetic engineering technologies for crop improvement in potato (Tejavathi and Indira, 2013; Yasmin *et al*., 2003). The lack of suitable *in vitro* regeneration systems limits further work like transformation and somaclonal variations of *P. edulis*. That is why the present study developed *in vitro* regeneration protocol for *P. edulis.* This creates new opportunity for improvement of this crop.

3 Objectives

3.1 General objective

- To develop *in vitro* regeneration protocol for *P. edulis* using leaf explant.

3.2 Specific objectives

- To optimize growth regulators concentration for optimal organogenic callus induction from leaf of *P. edulis.*
- To identify the optimal combination of growth regulators concentration for shoot regeneration.
- To investigate growth regulators concentrations for shoot multiplications that are generated from leaf derived calli.
- To acclimatize plantlets and evaluate their survival rate.

4 Materials and Methods

4.1 Plant material

In vitro propagated *P. eduils plants* (cultivar Holeta) were obtained from Addis Ababa University; Plant Biotechnology Laboratory. It was maintained by sub culturing every four weeks on MS basal medium with 1 mg/l BAP + 0.1 mg/l TDZ and 30 g/l sucrose using GA-7 Magenta and sealed with parafilm. The medium was gelled with 8 g/l agar and the pH was adjusted to 5.8 before autoclaving at 121 ^{0}C for 15 minute. Then thirty days old *in vitro* grown shoots were used for the study as explants.

4.2 Media preparation

4.2.1 Stock solution preparation

Murashige and Skoog (1962) basal medium was used throughout this research activity.

Full strength stock solutions of the media were prepared separately by weighing the recommended amount of macronutrients, micronutrients, vitamins and Fe-Na-EDTA and $FeSO_4$. 7 H_2O mixtures. Appropriate amount of each nutrient was weighed (Appendix 1) and dissolved in double distilled water by using magnetic stirrer. The stock solutions were poured in to plastic bottles and stored at -20 ℃ until use.

4.2.2 Plant growth regulators preparation

Plant growth regulators, 6-Benzyl amino purine (BAP), thidiazuron (TDZ), α-naphthaline acetic acid (NAA), Gibberellic acid (GA_3) and Indole-3-butyric acid (IBA) were used in this study and prepared by dissolving 1.0 mg of PGRs in 1.0 ml of double distilled water. The powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1M NaOH or HCl based on the requirement of the growth regulators (base for auxin and acid for cytokinins). After complete dissolution, the solution of each PGRs were poured into labeled volumetric flask and filled with double distilled water to the required volume. Then gently stirred and stored at 4 ℃ for short term use (maximum of two weeks).

4.2.3 Culture medium preparation

For callus induction, 50 ml/l macronutrient, 10 ml/l vitamins, 10 ml/l micronutrients and 10 ml/l iron EDTA, 30 g/l sucrose, different concentrations of auxin (NAA) and cytokinin (BAP) (Table 3) were mixed and allowed to dissolve by using magnetic stirrer. After the additions of PGRs, pH of medium adjusted to 5.8 using 1 M HCl or 1 M NaOH. Finally, 8 g/l agar was added and then the medium was autoclaved for 15 min at 121 ℃ and 105 KPa pressure. Twenty five milliliter of the medium was poured in to 90 mm diameter Petri dish in the laminar airflow cabinet and left for about 45 minute to solidify before the leaf explant was cultured on it. For shoot regeneration, shoot multiplication and rooting, the same conditions were used like that of callus induction. But, before autoclaving the medium was boiled on a microwave oven until the medium becomes clear and about 40 ml of the medium was poured into Magenta GA-7 culture vessel. Different concentrations of PGRs were also used for shoot regeneration (Table 4); shoot multiplication (Tables 5) and rooting (Table 6). Then after, all prepared media were stored at 4 ℃ for later use (about a week).

4.3 Callus induction

About 5 mm long young leaves were excised from *in vitro* grown shoots and wounded perpendicular to the midrib with scalpel. The leaves were cultured with adaxially position in contact with the medium. These explants were cultured on MS medium supplemented with different concentrations of NAA in combination with BAP, without PGRs was control. Ten leaves per Petri dish and 10 replications for each treatment (a total of 100 leaves) were used. The Petri dishes were properly sealed, labeled and placed in the incubator at 25±2℃. After four weeks, frequencies of callus induction were recorded (Table 3).

4.4 Shoot regeneration

Four-weeks-old calli were transferred to MS medium supplemented with different concentrations of BAP (0.0 mg/l, 0.1 mg/l, 0.5 mg/l, 1.0 mg/l and 1.5 mg/l) and TDZ (0.0 mg/l, 0.1 mg/l, 0.5 mg/l and 1 mg/l) for shoot regeneration. After a month, these calli were transferred to the same fresh MS medium. All the cultures were kept at 25±2 ℃ under dark condition until the shoots arose from calli. After six weeks, when shoots were regenerated from calli, the cultures were kept under dim light (20 µmol m^{-2} $s^{-1)}$ for one week and then transferred into full light of 40 µmol m^{-2} s^{-1} and 16 h photoperiod. After eight weeks, number of calli that produced shoots, number of shoots and shoot length per callus was recorded as presented in table 4.

4.5 Shoot multiplication

Regenerated shoots were cultured in Magenta GA-7 culture vessels containing 40 ml of the medium. The medium supplemented with different concentrations of BAP, TDZ and GA_3 were used for shoot multiplication either alone or in combination (Table 5). Eight shoots per Magenta and four replications for each treatment were used (a total of 32 shoots). Number and length of shoots per explant were recorded after four weeks.

4.6 Rooting

About 3-5 cm long shoots that were multiplied from shoots regenerated from leaf calli of *P. eduils* were cultured on MS basal medium supplemented with different concentrations of IBA and PGRs free medium was used as a control. Eight shoots per Magenta and four replications for each treatment were used (a total of 32 shoots). Finally, the cultures were placed randomly in the growth chamber. After four weeks, number and length of the roots were recorded.

4.7 Acclimatization

In vitro rooted shoots were removed from rooting medium and washed under running tap water. For *ex vitro* rooting, about 4 cm long microshoots were used. They were transferred to small pots filled with red soil, sand and compost in 2:1:1 ratio, respectively. Eight shoots per pot and thirteen replications were (a total of 104 microshoots) acclimatized for *in vitro* rooted and microshoots.

Pots containing both *in vitro* rooted plantlets and microshoots were covered with polyethylene bags. Polyethylene bags were partially removed after a week and completely removed after two weeks. The plantlets were watered every day using sprayer and kept for four weeks in glasshouse. After four weeks, number of survived plantlets were recorded and transferred to open sunshine. Finally, the number of plants that survived in the open sunshine was also recorded after three weeks.

4.8 Data analysis

Completely randomized design was used throughout this experiment (CRD). The one-way analysis of variance (ANOVA) was used to compute mean number of regenerated shoots, shoot multiplication, the number and length of roots and their survival rate in glasshouse. All data were analyzed at p ($\alpha<0.05$) using SPSS version 14 statistical software. Data were subjected to analysis of variance and variables that showed significant difference were compared by the LSD at 5 % probability.

5 Results

5.1 Callus induction

Callus induction was observed within four weeks of incubation from leaf explants on MS medium containing different concentrations and combinations of NAA and BAP. After three weeks of culture, different changes on leaf explants of *P. eduils* were observed. The callus thus obtained was compact and white in colour. The size and the shape of the explant also changed into dome shape calli (Fig.1 D). Callus induction was initiated from the wounded surface of the explants and finally covered the whole leaf. It has also been observed that the wounded areas of explants induced calli faster than unwounded areas of explants.

Among different combinations of PGRs, the maxium calli were recorded on MS medium supplemented with 1.5 mg/l and 2 mg/l NAA in combination with 1.0 mg/l and 0.5 mg/l BAP, respectively. The frequencies of induced calli on this combination were 100 %. However, all combinations of PGRs induced calli except the control (PGRs free medium) (Table 3). The rate of callus induction was significantly different among combination of plant growth hormones.

Table 3 Percentage of calli induced from leaf explants of *P. eduils*

Growth regulators (mg/l)		**Callus induction**
NAA	BAP	**%**
0.0	0	0
0.01	0.1	30
0.01	0.5	60
0.01	1.0	69
0.01	2.0	96
0.1	0.1	80
0.5	0.5	67
0.5	1.5	96
1.5	0.1	74
1.5	1.0	100
2.0	0.5	100

5.2 Shoot regeneration

After six weeks of culturing in shooting medium, shoot formation was observed from those calli that were induced on MS medium containing NAA (0.01, 0.5 and 1.5 mg/l) in combination with BAP (1.0, 0.5 and 0.1 mg/l). As shown in Table 4, most calli didn`t regenerate shoot. The frequency of shoot regeneration was influenced by the concentrations of NAA and BAP. The highest shoot regeneration frequency (46.6 %) was obtained on MS medium supplemented with 0.1 mg/l BAP in combination with 0.5 mg/l TDZ whereas the lowest frequency (6.7 %) was obtained on medium containing 0.5 mg/l

BAP in combination with 0.5 mg/l TDZ. The highest mean shoot number per callus (1.66±1.04) was obtained on MS medium supplemented with 1.5 mg/l BAP in combination with 1.0 mg/l TDZ whereas the highest mean shoot length (0.63±0.19 cm) was produced on MS medium containing 0.5 mg/l BAP in combination with 0.1 mg/l TDZ.

Table 4. Percentage of regeneration, mean number and height of regenerated shoots from calli of *P. eduils* values given as mean ± SE

Conc. of PGRs (mg/l)		Percentage of Regeneration	Mean shoot number per callus	Mean shoot height (cm)
BAP	TDZ	%		
0	0	0	$0±0^{bcd}$	$0±0^{c}$
0.1	0.5	46.6	$0.46±0.92^{ac}$	$0.60±0.15^{ab}$
1.5	1	13.3	$1.66±1.04^{a}$	$0.09±0.04^{c}$
0.5	0.1	30	$1.36±0.87^{ab}$	$0.63±0.19^{a}$
1.5	0.1	13.3	$0.26±0.17^{ad}$	$0.25±0.12^{c}$
0.5	0.5	6.7	$0.13±0.10^{bcd}$	$0.06±0.05^{c}$

Means within each column connected by the same superscript (a-d) are not significantly different at 5 % probability level.

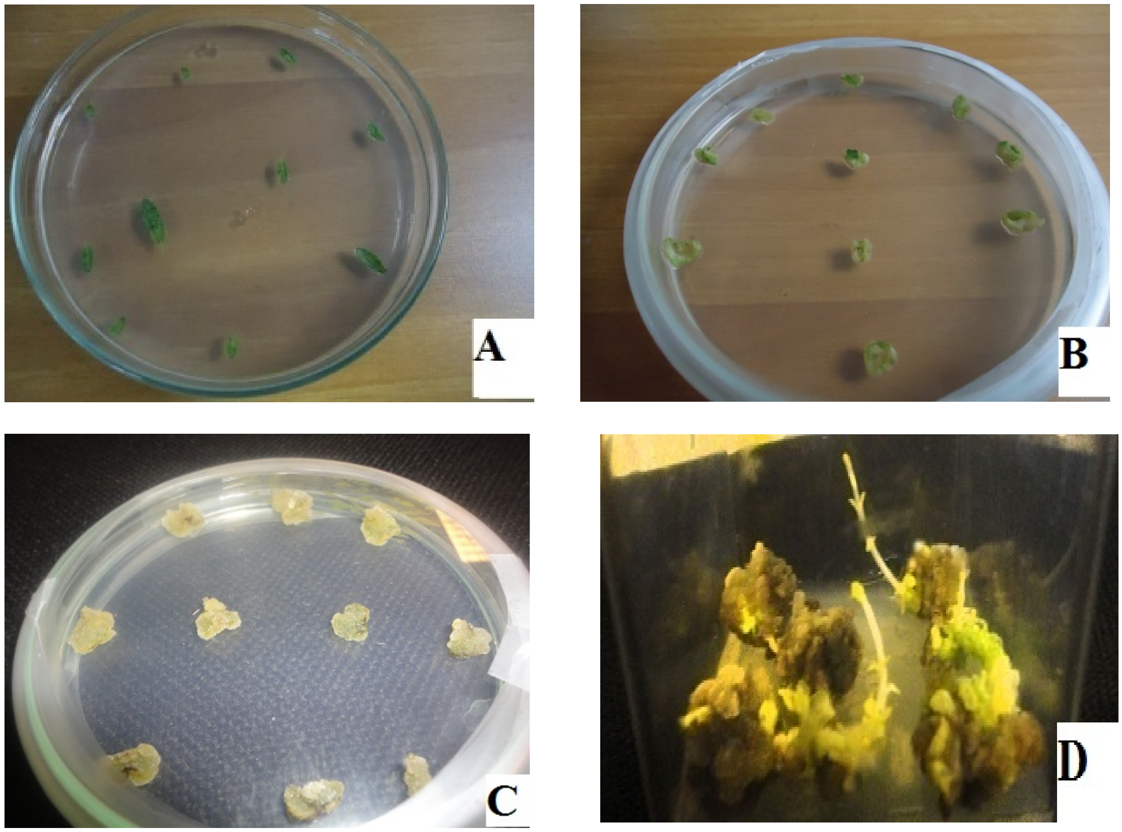

Figure 1. Calli formation and shoot regeneration of *P. edulis* from leaf explants. (A) Leaf explants cultured adaxially on MS medium supplemented with 0.01 mg/l NAA in combination with 1.0 mg/l BAP; (B) Three-week-old induced calli; (C) Four-week-old calli; (D) Six-week-old regenerated shoots after transferred to MS medium supplemented with 0.1 mg/l BAP combined with 0.5 mg/l TDZ.

5.3 Shoot multiplication

Shoot number and length were highly influenced by concentrations and types of PGR supplemented. The maximum shoot multiplication medium from the treatment used and better stage of multiplication that provided higher number of shoots was obtained. Different concentration of auxin and cytokinins used alone or in combination, resulted in different response on shoot multiplication medium (Table 5). Among all treatments used, average mean number of shoots per explant (58.68±6.57) was recorded on MS medium supplemented with 0.5 mg/l BAP in combination with 0.4 mg/l GA_3. Maximum number (129) of shoots per explant was also produced in these combinations. The multiplication rate of *P. edulis* on a medium containing BAP in combination with GA_3 was relatively higher than that of other PGRs, either used alone or in combinations. Medium containing 0.2 mg/l BAP + 0.5 mg/l GA_3, 0.4 mg/l BAP + 0.2 mg/l GA_3, and 1.5 mg/l BAP + 0.1 mg/l GA_3 produced better number of shoots than other treatments used.

The maximum mean shoot length (3.90±0.19 cm) was observed on MS medium supplemented with 0.2 mg/l BAP in combination with 0.5 mg/l GA_3. Medium containing 0.1 mg/l BAP + 1.5 mg/l GA_3 and 0.4 mg/l BAP + 0.2 mg/l GA_3 showed the second largest shoot length (3.67±0.18 cm).

Table 5. Mean number and length of shoots induced on MS medium containing different concentrations of PGRs (mean values are indicated as ± SE)

Growth regulators (mg/l)			Mean No. of shoots/explant	Mean shoot height (cm)
BAP	GA_3	TDZ		
0	0	0	3.93±0.49^{ghi}	2.06±0.18^{fh}
0.1	1.5	0	11.31±0.96^{eg}	3.67±0.18^{ab}
0.2	0.5	0	23.34±2.99^{d}	3.90±0.19^{a}
0.3	1	0	15.53±2.14^{e}	3.26±0.17^{bcd}
0.4	0.2	0	42.28±5.27^{b}	3.67±0.12^{ac}
0.5	0.4	0	58.68±6.57^{a}	2.98±0.16^{de}
1	0.5	0	17.81±2.66^{e}	2.71±0.24^{ef}
1.5	0.1	0	34.90±3.95^{c}	2.82±0.15^{de}
0.5	0	0.01	16.28±1.51^{e}	1.96±0.12^{ghi}
0.5	0	0.1	15.90±1.57^{e}	1.75±0.09^{ghl}
0.5	0	1	6.93±0.73^{fghi}	3.14±0.21^{bce}
0.5	0	0	5.40±0.97^{fghi}	1.83±0.14^{ghk}
0.8	0	0	7.50±0.78^{fghi}	2.09±0.15^{fg}
2	0	0	11.21±0.77^{ef}	1.32±0.08^{jkln}
3	0	0	7.09±0.83^{fghi}	1.81±0.59^{ghj}
0	0	0.5	10.28±1.05^{ei}	1.32±0.08^{ijklm}
0	0	1	10.06±1.59^{eh}	1.02±0.09^{mn}
0	0	2	3.96±0.69^{ghi}	0.44±0.06^{o}

Means connected by the same superscript letters (a-o) in the same column are not significantly different at 5 % probability level.

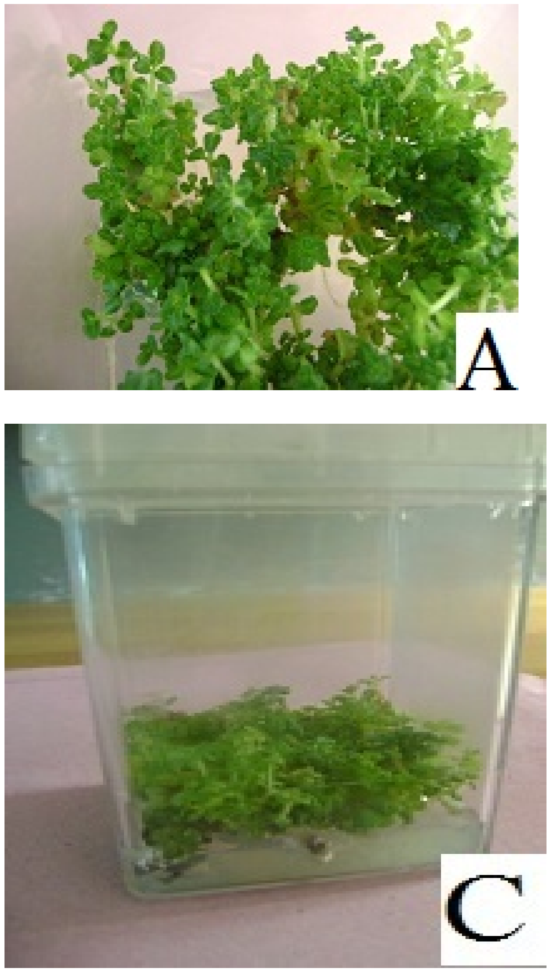

Figure 2. Shoot multiplication from regenerated shoots. (A) 0.5 mg/l BAP + 0.01 mg/l TDZ; (B) 0.4 mg/l BAP + 0.2 mg/l GA_3; (C) 2.0 mg/l BAP

5.4 Rooting and acclimatization

5.4.1 *In vitro* rooting

The shoots that were derived from calli cultured in MS basal media supplemented with different concentrations of IBA showed different rooting responses. Among the nine different concentrations of IBA used, maximum mean number of roots per plantlet (10.15±0.95) and mean root length (5.06±0.35 cm) was recorded on MS medium supplemented with 2.0 mg/l and 0.6 mg/l IBA, respectively (Table 6).

Table 6. Mean number and length of roots of *P. eduils* obtained on MS medium supplemented with different IBA concentrations, values given as mean ± SE

IBA (mg/l)	Mean number of roots per explant	Root length (cm)
0	7.78 ± 0.96^{bcdef}	1.57 ± 0.19^{e}
0.1	9.03 ± 1.03^{ad}	2.65 ± 0.17^{cd}
0.2	8.37 ± 0.72^{af}	3.16 ± 0.40^{bd}
0.3	0.68 ± 0.25^{h}	0.59 ± 0.27^{e}
0.4	9.09 ± 7.83^{ac}	3.53 ± 0.35^{b}
0.6	9.93 ± 0.70^{ab}	5.06 ± 0.35^{a}
1.0	3.59 ± 0.83^{g}	1.57 ± 0.40^{e}
1.5	8.03 ± 0.82^{ae}	4.31 ± 0.31^{a}
2.0	10.15 ± 0.95^{a}	3.26 ± 0.30^{bc}

Means within each column connected by the same superscript (a-f) are not significantly different at 5 % probability level.

Figure 3. Shoots of *Plectranthus edulis* rooted on rooting media containing different concentrations of PGRs. (A) 0.3 mg/l IBA; (B) 0.6 mg/l IBA and (C) 2 mg/l IBA

5.4.2 *Ex vitro* rooting

After acclimatization, all the survived shoots were developed root and induced a mean of 11.86±0.52 (*in vitro*) and 8.42±0.70 (microshoots) roots. Its maximum mean root length was 6.45±0.18 and 5.03±0.31 cm from *in vitro* and microshoots, respectively. Number of roots per shoot and root length was significantly different from results of all other MS rooting medium. By comparing *in vitro* treated ones, *ex vitro* grown shoots had very good morphological appearance (Fig. 4) and highest number of mean root (11.86±0.52) and length (6.45±0.18 cm).

Table 7. Mean number and length of roots of *P. eduils* obtained after acclimatization, values given as mean ± SE

	Mean number of roots per explant	Root length (cm)
In vitro	11.86 ± 0.52^{a}	6.45 ± 0.18^{a}
Ex vitro (microshoots)	8.42 ± 0.70^{b}	5.03 ± 0.31^{b}

Means within each column connected by the same superscript (a and b) are not significantly different at 5 % probability level.

5.4.3 Acclimatization

All (100 %) of plantlets that were rooted under *in vitro* conditions survived. However, among the microshoots that acclimatized in the glasshouse, only 78 % of the plantlets were survived (Fig. 4). The plants grew well in the pots as well as in the open sunshine.

Figure 4. Stages of acclimatization for *in vitro* and microshoots. (A, E) Plantlets covered with polythene plastic bags; (B, F) Four weeks after acclimatization; (C, G) Rooted shoots after four weeks; (D, H) Three weeks grown in the external environment.

6 Discussion

6.1 Callus induction

Among the 11 different PGRs combinations used, the highest (100 %) callus induction was observed on MS medium supplemented with 1.5 mg/l NAA in combination with 0.5 mg/l BAP and 2.0 mg/l NAA in combination with 0.5 mg/l BAP. All combinations of PGRs induced calli except PGRs free medium. The percentage of callus induction was significantly varied between different concentrations of PGRs.

In the present study, callus was induced on the leaves at the sites wounded with scalpel perpendicular to the midrib and then the whole leaf was covered by callus. Similar results were reported by Sharma *et al*. (2012) that callus was initiated from longitudinally injured surface of the leaf and also proliferated from cut end of *Ephedra gerardiana* explants. Tileye Feyissa *et al.* (2005) also reported that wounding promoted earlier callus formation. This might be due to wounded area traps more nutrients so that callus induction was faster than unwounded explants. However, it is critical to ensure that, in such cases, wounding does not negatively affect regeneration potential (Zimmerman and Fordham, 1985).

In this study, most of the combinations of NAA and BAP resulted in better callus induction (70-100 %) (Table 3). Hence, the frequency of callus induction varies depending on the concentrations of PGRs, specifically high concentration of NAA highly promoted callus induction on *Dendrobium candidum*(Zhao, 2008), and *Tageteserecta* (marigold) plants (Hussain and Latif, 2012). The present study is in agreement with these reports.

According to Razdan and Mattoo (2004), callus was usually induced using MS medium supplemented with both auxin and cytokinin hormones. In the present study, all combinations of auxin (NAA) and cytokinin (BAP) induced better calli. The leaf explants grown on all media containing PGRs expanded after 2 weeks and produced calli after 3 weeks, but the control explants on growth regulator free medium did not show expansion and callus formation. Present result is in agreement with the report of Tejavathi and Indira (2013), in which BAP in combination with NAA promoted callus in *Drymaria cordata* plant. Martel *et al*. (1992) on Solanum tuberosum L. cv. Sebago and Yasmin *et al.* (2003) on potato plantlets also reported that NAA was essential for callus induction and the amount of callus induction increased with increased concentration of NAA and BAP in potato.

According to Yasmin *et al*. (2003), percentage of callus induction was highest (95 %) in MS medium containing 2.0 mg/l BAP in combination with 2.5 mg/l NAA from leaf explants of potato. Other similar responses were reported by Onamu (2012), where 100 % of callus was induced using nodal explants of potato (*Solanum tuberosum* L.) cultivars grown in Mexico with 4.0 mg/l BAP in combination with 1.0 mg/l NAA.

Contrasting results were reported on tissue culture of *Solanum melongena* L., where callus formation was optimum when MS medium was supplemented with low concentration of NAA and high concentration of BAP (Ray *et al.*, 2011). Arumugam *et al.* (2009) observed that callus induction rate declined gradually when the concentrations of cytokinin and auxin were either increasing or decreasing from the optimum on *in vitro* plant regeneration from immature leaflets of *Acacia confusa* Merr.

6.2 Shoot regeneration

Cytokinins are known to stimulate plant cell division, release of lateral bud dormancy, and induction of adventitious bud formation and growth of lateral buds (Coello *et al.*, 2010). In this study, shoots were observed only from those calli induced from MS medium supplemented with 0.01 mg/l, 0.5 mg/l and 1.5 mg/l NAA in combination with 1.0 mg/l, 0.5 mg/l and 0.1 mg/l BAP, respectively. However, the calli that were formed on other combinations of PGRs didn't regenerate shoot. Results of current study showed that best response (46.66 %) towards multiple shoot regeneration from callus obtained from leaf as explants was obtained on MS medium containing 0.1 mg/l BAP combined with 0.5 mg/l TDZ. However, shoots were not regenerated from calli that were cultured on PGRs free medium. Similar result was reported by Yasmin *et al*. (2003) who stated that PGRs free medium did not regenerate shoots on leaf and intermodal segment of potato.

The result of this study revealed that there was no relationship between callus formation ability and shoot regeneration. Furthermore, shoots were regenerated from calli that were derived from longitudinally wounded leaves. Ganesan and Jayabalan (2005) pointed out that leaf explants exhibited a low ability to form callus, but leaf derived calli regenerated shoots at a relatively high percentage on shoot tips in cotton plant. Additionally, whole leaf explants wounded along the midribs were more regenerative than stem in leaves of wild pear (Bhagwat and Lane, 2004).

Khalafalla *et al.* (2010); Tasew Getu (2011) reported that two shoot per calli was the maximum number of shoots observed on potato (*Solanum tuberosum* L.) cultivar

‘Almera’ and sweet potato variety ‘Beletech’, respectively. This was not consistent with the present study as 30 shoots per callus was the maximum number of shoots recorded on *P. eduils* plant.

6.3 Shoot multiplication

A report by Roca *et al*. (1978) showed that rapid shoot multiplications were obtained on MS medium supplemented with BAP, GA_3 and NAA alone or in combinations on propagation of potatoes. In the present study, among different multiplications media used, the highest mean shoot number per explant was 58.68±6.57 on MS medium supplemented with 0.5 mg/l BAP and 0.4 mg/l GA_3. Duraisamy *et al.* (2012) also reported that maximum mean number of shoots per explant were obtained (6.3±0.09) using MS medium supplemented with (0.02 mg/l) GA_3 + (0.1 mg/l) BAP on apical meristem cultures of cassava.

Severe chlorosis was observed in the plants cultured on medium containing 1.0 mg/l GA_3 (Rabbani *et al.*, 2001). But, the toxic effect of GA_3 was minimized when it was used in combination with BAP. Combination of 0.5 mg/l GA_3 and 0.5 mg/l BAP exhibited best result with almost 1.3 and 1.2 fold increase in shoot numbers and plant height on *in vitro* regeneration of *Solenostemon scutellarioides.* This might be the reason for the present study when maximum mean number of shoots per explant (58.68±6.57) and mean height (3.90±0.19 cm) was recorded by combining BAP with GA_3. Unlike the result of Tasew Getu (2011) who showed that more sweet potato shoots per explant (2.40±0.113) was obtained on MS medium supplemented with 1.0 mg/l BAP than those cultured on medium containing GA_3 combined with BAP. Badoni and Chauhan (2009) also reported that GA_3 and NAA combination is best for shoot regeneration and multiplication of potato cv. 'Kufri Himalini' in comparison to using combination of Kinetin and NAA.

Mesfin Tsegaw (2012) obtained the highest mean number of shoots per explant (7.2) of *P. edulis* on MS medium supplemented with 1.0 mg/l KIN + 0.1 mg/l NAA. Teslim Yimam (2013) reported that highest mean number of axillary shoots per explant (5.85±2.17 and 6.07±2.30) was obtained on MS multiplication medium (solid and liquid medium, respectively) containing 1.5 mg/l BAP + 0.5 mg/l TDZ in *in vitro* propagation of *P. eduils*. However, in the present study, highest mean shoot number per explant (58.68±6.57) was obtained on MS medium supplemented with 0.5 mg/l BAP + 0.4 mg/l GA_3.

On the other hand, Teslim Yimam (2013) reported highest mean number of shoots per explant (5.85±2.17) on solid medium supplemented with 0.5 mg/l TDZ in combination with 1.5mg/l BAP. However, in the present study, using lower concentration (0.5 mg/l BAP + 0.01 mg/l TDZ), produced better mean number of shoot per explant (16.28±1.51). Hence, this difference might be, the shoots obtained via callus may be more juvenile and are suitable for mass propagation. A similar response was mentioned by Zhao (2008) that shoots derived from callus exhibited great importance for mass propagation in many species.

6.4 Rooting and acclimatization

6.4.1 Rooting

In the *in vitro* rooting, the highest mean number of roots per explant (10.15±0.95) and mean length (5.06±0.35 cm) were obtained on MS medium supplemented with 2.0 mg/l and 0.6 mg/l IBA, respectively. All rooting medium, including growth regulators-free medium, induced rooting.

Shahzad *et al*. (2009) described that IBA rooting medium was better than IAA and NAA. Specially, NAA supplemented medium induced thin delicate roots with slow growth and resulted in some callus formation which is considered to be undesirable for *ex vitro* establishment that was done in *Sansevieria cylindrical*. In their result, maximum number of roots (3.5±0.18) and root length (6.5±0.14 cm) were obtained on 5 μM IBA incorporated into half strength MS medium.

In the present study, most of the rooting media resulted in more than 90% rooting. Khalafalla *et al.* (2010) reported that all concentrations of IBA used with both full-MS and half-MS strengths on potato (*Solanum tuberosum* L.) cultivar 'Almera' induced 100% rooting.

Another report by Mesfin Tsegaw (2012) showed that a maximum number of mean roots per explant (3.23) was obtained on MS medium supplemented with 1.0 mg/l IBA in Holeta locals of *P.eduils* plant and resulted in 95 % rooting. In the present study, equal number of mean roots per explants (3.23±0.82) was recorded but 100 % rooting was obtained on medium containing 1.0 mg/l IBA. However, maximum mean number of roots per explant (10.15±0.95) was obtained at 2.0 mg/l IBA.

In *ex vitro* rooting, Mesfin Tsegaw (2012), recorded that maximum mean number of roots (6.17) and length (3.3 cm) in Holota locals of *P. eduils*. In present study, the overall best result in rooting was observed in *ex vitro* rooting condition. The highest mean number of roots (11.86±0.52) and length (6.45±0.18 cm) was obtained. The reason might be, roots formed in agar have very small number of root hairs and act like "water roots" (Hollings, 1965).

The result of this study also shows the fact that acclimatization of microshoots of *P. edulis* can efficiently replace *in vitro* rooting works. Therefore, using microshoots rooting makes the protocol very economical in *P. eduils* plant.

6.4.2 Acclimatization

Successful acclimatization depends primarily on the rate of root formation. However, in the present study both microshoots and *in vitro* rooted shoots were evaluated for their survival rates in the glasshouse and external environment.

The plantlets produced *in vitro* are highly susceptible to the *ex vitro* condition until they develop adaptation mechanisms to cope with the environmental stress. In the present study, 100 % survival rates of the plantlets were observed in the glasshouse as well as the external environment. The result is consistent with the work of Tasew Getu (2011) on sweet potato and Mesfin Tsegaw (2012) and Teslim Yimam (2013), *P. eduils*. All of them reported 100 % survival in the glasshouse.

Acclimatization of microshoots was evaluated in the glasshouse and then transferred to the external environment. Among the total plantlets used, 78 % of plantlets were survived in the glasshouse as well as in the open sunshine. The reason could be the ability of *P. eduils* to propagate through vegetative means.

In the present study, 78 % of the microshoots survived without any auxin treatment. However, Mesfin Tsegaw (2012) demonstrated that *ex vitro* rooting by immersing the microshoots into 5 mg/l of IBA for 5 minutes before acclimatization resulted in 76.6 % survival of *P.eduils* (Welayta local). The present study reduces the cost of rooting hormones.

7 Conclusion

- The highest percentage of callus induction was 100 % from leaf explants of *P. eduils* on MS medium supplemented with 1.5 mg/l NAA + 1 mg/l BAP and 2.0 mg/l NAA + 0.5 mg/l BAP.
- Maximum regeneration frequency (46.6 %) was recorded on MS medium containing 0.1 mg/l BAP combined with 0.5 mg/l TDZ. However, there is no correlation between callus formation ability and shoot regeneration.
- The shoots induced from calli could be further used to induce shooting and rooting *in vitro*. The combination of (0.5 mg/l) BAP and (0.4 mg/l) GA_3 showed best response for obtaining maximum mean shoots (58.68±6.57) proliferation than other multiplications media.
- Maximum mean roots/explant (10.15±0.95) was obtained on MS medium containing 2.0 mg/l IBA.
- All *in vitro* rooted (100%) and 78 % of microshoots were survived in the glasshouse and open sunshine.
- The present study has standardized regeneration protocol for callus induction of the tuber plant *P. eduils* from leaf explants. Apparently, indirect *in vitro* regeneration is the first work on this tuber plant.

8 Recommendations

- Different explant sources other than leaf should be tested for callus induction and shoot regeneration, in order to compare their callus induction frequency and shoot regenerating ability.
- Further study on tuberization, direct regeneration and somatic embryogenesis of these tuber plants on different local cultivars should be tested by using different growth regulators.
- Agronomic performance of *P. eduils* derived from tissue culture regenerated plants should be investigated.
- Much more shoots could be derived from shoots derived from calli than those obtained through micropropagation from meristem or shoots. Therefore, those plants obtained from *in vitro* regeneration through callus phase should be evaluated for their genetic stability as they may show somaclonal variation.
- Somaclonal variation and genetic transformation should be tested.

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10 Appendices

Appendix 1 Full MS basal medium stock solution composition.

Micronutrients	g/l	Macronutrient	g/l	Vitamin	g/l
Fe–Na-EDTA	4	NH_4NO_3	16.5	Nicotinic acid (NaOH)	1.00
$ZnSO_4$. 7 H_2O	0.86	KNO_3	19	Glycin (glycocoll)	0.2
H_3BO_3	0.62	$CaCl_2$. $2H_2O$	4.4	Nicotinic acid (NaOH)	0.05
*$MnSO_4$. H_2O	1.69	$MgSO_4$. $7H_2O$	3.7	Pyridoxin (B6)	0.05
*$MnSO_4$. $4H_2O$	2.23	KH_2PO_4	1.7	Thiamin (B1)	0.01
$CuSO_2$. 5 H_2O	0.025				
KI	0.083				
Na_2MoO_4. $2H_2O$	0.025				
$CoCl_2$. 6 H_2O	0.25				

* are alternatives

Appendix 2 Data showing number of explants which gave multiple shoots

Treatment code No.	No. of explants which gave multiple shoots	Percentage (%)
1	22	68.75
2	32	100
3	32	100
4	32	100
5	32	100
6	32	100
7	32	100
8	31	96.87
9	32	100
10	32	100
11	30	93.75
12	26	81.25
13	27	84.37
14	32	100
15	30	93.75
16	32	100
17	29	90.62
18	20	62.5

32 explants per treatment were used.

Appendix 3 Data showing number of explants which gave root

Treatment code No.	No. explants which gave multiple roots.	Percentage (%)
1	30	93.75
2	9	28.12
3	31	96.66
4	32	100
5	29	96.87
6	32	100
7	32	100
8	20	62.25
9	32	100

32 explants per treatment were used.

Appendix 4 Number of shoots (*in vitro* rooting and microshoots) survived in the glasshouse.

	Shoots survived	Percentage (%)
In vitro rooting	104	100
microshooting	78	78

104 explants were used for acclimatization in the glasshouse.

Declaration

I, Nitsuh Aschale Yenesew, hereby declare that, this thesis is my original work. It has never been submitted for a degree in any other institution and that all sources of materials used for the thesis have been fully acknowledged. No part of this thesis may be produced, stored in any retrieval system, or transmitted in any form, or by any means (e.g. electronics, mechanical, photo copying, recording or otherwise) without prior permission of the author or Addis Ababa University in that behalf.

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This thesis has been submitted for examination with my approval as the research advisor.

Advisor: ______________________Signature:__________ Date: ____________________