ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

INSTITUTE OF BIOTECHNOLOGY

Micropropagation of *Plectranthus edulis* (Vatke) Agnew from Meristem Culture

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

By

Mesfin Tsegaw

Addis Ababa, Ethiopia

December, 2012.

Abstract

Micropropagation of Oromo Dinich (*Plectranthus edulis* (Vatke) Agnew) from meristem culture

Mesfin Tsegaw

Addis Ababa University, 2012

Plectranthus edulis (*syn.* Coleus edulis) *is a tuber-bearing labiate species originated from Ethiopia. It has been grown and used as a major source of food in many parts of Ethiopia. It is one of the traditional and under-utilized root crops. Although this plant is being promoted to be used by most people in the country, there is shortage of pathogen free planting material.* In vitro *propagation of* P. edulis *has immense value in rapid production, eradication of pathogens and conservation of genetic diversity. The objective of this study is to develop micropropagation protocol for* P. edulis *using meristem as explant.* P. edulis *tubers collected from two growing areas were planted in glasshouse to serve as explants donor. For this experiment solid MS medium supplemented with* GA_3*, NAA and different BAP concentrations were used to initiate the growth of shoots from meristems. Shoots were multiplied using MS medium containing different concentrations and combinations of BAP and Kinetin alone and in combination with NAA. The rooting experiment was done using half and full strength MS medium containing 1.0 mg/l IAA, IBA or NAA, and* ex-vitro *rooting were also tested by dipping micropropagated shoots in 5.0 mg/l IBA for 5 minutes before it was transferred to small pots in glasshouse. Among the various concentrations of BAP, 0.1, 0.5, 1.0, 2.0, and 5.0 mg/l tested, shoot induction was found to be effective in 1.0 mg/l BAP combined with 1.0 mg/l* GA_3 *and 0.1 mg/l NAA. Out of single nodal shoot cultured in different multiplication medium, those cultured in MS medium supplemented with 1.0 mg/l KIN and 0.1 mg/l NAA from Holeta, and 3.0 mg/l KIN and 0.05 mg/l NAA from Welayta showed the highest number of multiple shoot (7.2 and 6.2, respectively).* In vitro *developed shoots were rooted best (with mean of 6.17 and 5.43 roots from Holeta and Welayta, respectively)* ex vitro *by dipping in 5.0 mg/l IBA for 5 minutes. Second best rooting was on half strength MS medium containing 1.0 mg/l IBA. All plants from Holeta and 96 % of Welayta survived in glasshouse two weeks of acclimatization and no aberrant plants were observed.*

Key Words: *Ex vitro*, GA_3, Holeta, meristem culture, kinetin, shoot multiplication, Welayta

Acknowledgements

My deepest gratitude goes to my advisor, Dr. Tileye Feyissa, for his continuous, unreserved, constructive guidance and support as well as providing the necessary resources unlimitedly. I am very fortunate to have worked under his guidance and am proud to have studied in his laboratory.

My heartfelt gratitude goes to Prof. Legesse Negash for providing me the lab space and materials while conducting my study. I would like to extend my gratefulness to Addis Ababa University, School of Graduate Studies, for providing me financial support.

I would like to extend my gratefulness to Hawassa University for giving me the opportunity to pursue a great education. I would like to express my gratitude to staffs of IBC. They have been helping me by their explicit knowledge and experience in identification of my research area.

I would like to thank Addisu Fekadu for his support in getting importance peoples who are willing to give seed tubers in his place and Endrias for generously providing seed tubers of *P. edulis*. Without your contribution this would not have been possible.

I am grateful to Idris Youna for assisting me in meristem isolation at the beginning of my work and Tewodros Tesfaye for his guidance throughout my work. My thanks to all of my department and lab colleagues, you were a source of support, inspiration and a lot of fun.

Finally, I am indebted to my family, whose commitment to my studies was invaluable. Thank you Hani for all that you sacrificed, Mom and Dad for giving me your all rounded support and love.

Contents

List of Figures

List of Tables

List of Appendices

List of Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
ANOVA	Analysis of Variance
BAP	6-benzyl Amino Purine
EDTA	Ethylene Di-Amine Tetra acetic Acid
GA_3	Gibberrelic Acid
IAA	Indole 3- Acetic Acid
IBA	Indole 3-Butyric Acid
IBC	Institute of Biodiversity Conservation
IITA	International Institute of Tropical Agriculture
ISSR	Inter Simple Sequence Repeat
KIN	Kinetin
LSD	Least Significant Difference
MAFF	Ministry of Agriculture, Forestry and Fisheries of Japan
MS	Murashige and Skoog
NAA	α-naphthaline Acetic Acid
PGRC	Plant Genetic Resource Center
PGRs	Plant Growth Regulators
RAPD	Randomly Amplified Polymorphic DNA
RH	Relative Humidity

1. Introduction

Plectranthus edulis (Vatke) Agnew synonymous *Coleus edulis* is an indigenous root crop in Ethiopia and it has important place in the diet of the population (PGRC, 1996; IBC, 2005). *P. edulis* is grown and used as a major source of food in many parts of Ethiopia and is liked as a tasty source of carbohydrates (Mulugeta Taye *et al.,* 2007). It is one of the major crops cultivated in Oromia region together with coffee, fruits (mango, papaya, avocado, lemon, and orange), maize, teff (*Eragrostis teff*), anchote (*Coccinia abyssinica*) (Bula Sirika, 2011).

The genus *Plectranthus* has been distributed all over the tropical and subtropical regions of India, Pakistan, Sri Lanka, Tropical East Africa, Brazil and Egypt (Sunilkumar, 2005). It occurs both as wild and cultivated species in Ethiopia. However, *P. edulis* is one of the native tuber crops in Ethiopia (IBC, 2005). The cultivated species is grown in the wetter south and south western of Ethiopia whereas the wild species are found throughout the country (PGRC, 1996). It is believed that *P. edulis* sometimes serves as medicinal plant, eating the boiled root can avoid loss of appetite (Moa Megersa, 2010).

Root and tuber crops are present in the diet of people almost all over the world, the best known being the potato (*Solanum tuberosum* L.), sweet potato (*Impeoa batatas*) and cassava (*Manihot esculenta* Crantz), which have different origin. But there are several species of roots and tubers which originated in Ethiopia and which are still of importance to the people, such as Oromo Dinich (*Plectranthus edulis*) and Anchote (*Coccinia abyssinia*) (Dandena Gelmesa, 2010). These species are little known to farmers and investigators outside the growing region. They are species that have perhaps not been fully exploited or whose potential has not been fully taken advantage of, possibly because their cultivation is restricted and their use localized. Some of these species

have a nutritive value and other characteristics that make them very promising candidates for more extensive use. For example, *P. edulis* have a high energy value (Yeshitila Mekbib, 2007). The Livingstone-potato (*Plectranthus esculenta*) which is considered an untapped indigenous tuber crop of Nigeria has been paid no attention while it contributes significantly to nutritional security in some communities (Okereke, 2012).

Many locally very important root and tuber species "appear doomed to extinction" unless appropriate action is taken (MAFF Research Council, 1994). According to IBC (2005) *P. edulis* is one of the horticultural species, which are under constant threat of genetic erosion from deforestation and habitat destruction, expansion of farm and grazing lands, and problems related to land use change.

Padulosi *et al.* (2002) also indicated that the general decline of underutilize crop may erode the genetic base and preventing the use of distinctive useful traits in crop adaptation and improvement. *P. edulis* growers have specialized on few cultivars that they thought would meet their needs best. At least six local cultivars currently growing in area of Ethiopia where previous studies was made and all of them had been selected by farmers. There is, however, evidence of ongoing genetic erosion, which has resulted in the complete loss of some local cultivars (Yeshitila Mekbib, 2007). These are again mainly caused by rural poverty, population pressure, and changes in traditional agricultural production systems. Therefore, whatever cause does it has genetic erosion was clearly the result of production decline of this plant.

There is a tremendous untapped potential for Ethiopia to exploit the rich and diverse plant genetic resources underutilized root and tuber crops. However, in spite of their potential for food security and export, very little research has so far been done to improve the productivity of this

important crop category (IBC, 2007). The understanding of the causes behind the low level of use and/or neglect of a crop is however what is ultimately needed in order to design an appropriate strategy to address its improvement (Padulosi *et al.,* 2002).

The major production constraints identified by Mulugeta Taye (2008) are the shortage of seed tubers and the poor storability of the tubers. It is recommended to initiate research on seed production of *P. edulis* and on the effect of seed quality on the performance of the crop grown from that seed (Mulugeta Taye, 2008). Tissue culture may provide a method by which *P. edulis* growers can continually acquire large numbers of disease-free planting stock.

As tuber crop, there was report on the occurrence of diseases in *P. edulis* in some growing areas (Mulugeta Taye, 2008). Like other root and tuber crop, it is propagated vegetatively, and hence it is susceptible to diseases that are carried from one generation to the next through infected planting material. Vegetatively propagated crops are inherently more susceptible to the maintenance, increase, and dissemination of both systemic and nonsystemic diseases than are the sexually reproduced crops using true seed as planting material. The systemic diseases-viruses, viroids, and mycoplasma pathogens, as well as several bacteria are the most devastating in terms of yield loss for the root and tuber crops (Bryan, 1983).

The application of plant tissue culture offers valuable ways to overcome many of the problems that's found in natural propagation. For instance, meristem cultures were used to maintain and propagate cassava cultivars *in vitro* and to produce clean planting material free of viruses and other diseases (Puonti-Kaerlas, 1998). Meristem culture can be combined with thermotherapy to improve the elimination rate of a number of viruses and bacteria, and it also allows the use of large meristem explants up to 0.8 mm (Kartha and Gamborg, 1975).

Increased use of rapid multiplication techniques is enabling scientists to produce large amounts of pathogen-free material. Because most rapid multiplication techniques in root and tuber crops involve the use of aerial portions of the plant, contact with soil and tuber portions is broken and most nonsystemic pathogens and pests can be eliminated (Bryan, 1983). Meristem culture can even improve the technique by eliminating systemic pathogens present in the donor plant, from cultures (Honda *et al.,* 2001; Stepan, 1990).

In vitro propagation has several advantages over the conventional method of propagation. Some of this are starting with very small pieces of plant (explants), require small amount of space and produces virus free plant material. For example meristem cultures were used to produce plants free from cassava mosaic virus, when explants smaller that 0.4 mm was used (Puonti-Kaerlas, 1998). As the rate of propagation is much greater than the conventional propagation many more plants can be produced in a given time. This may enable newly selected varieties to be made available quickly and widely and numerous plants to be produced in a short while (George and Debergh, 2008). Moreover, having disease-free *in vitro* culture can serves as source of material for *in vitro* germplasm conservation which is an alternative for field collections (Puonti-Kaerlas, 1998).

A major advantage of working with meristems is the high probability of excluding pathogenic organisms, present in the donor plant, from cultures. The culture conditions are controlled to allow only organized outgrowth of the apex directly into a shoot, without the formation of any adventitious organs, ensuring the genetic stability of the regenerated plants (Ivan *et al.,* 2010).

The growth and proliferation of axillary shoots in shoot cultures is usually promoted by incorporating growth regulators (usually cytokinins) into the growth medium. Most often such a

treatment effectively removes the dominance of apical meristems so that axillary shoots are produced, often in large numbers. These shoots are used as miniature cuttings for plant multiplication (George and Debergh, 2008). Most commonly, shoots of axillary origin are genetically identical to the parent plant, whereas there is a probability that those regenerated from callus may differ in one or more characters.

Despite the problems, farmers in growing areas want to continue growing and consuming the tubers with their families and most of them want to increase the amount of area for *P. edulis* production (Mulugeta Taye, 2008). However, *P. edulis* is an orphan crop in national and international research; research to overcome the production constraints in this crop does not exist (Mulugeta Taye, 2008). There were only few researches on agronomy of the plant and there is no report on *in vitro* culture of *P. edulis*. *In vitro* method of vegetative multiplication of *P. edulis* will have considerable benefits for its availability as planting material, in the grower and germplasm conservation (since maintenance of plant germplasm requires the use of disease-free stocks). Therefore, the aim of this study is to develop micropropagation protocol for *P. edulis* using meristem as explant.

2. Literature Review

2.1. *P. edulis*: Description, origin and distribution

Plectranthus edulis (Vatka) Agnew (1974) species is variously known in different parts of Ethiopia as 'Dinicha Oromo', meaning "potato of the Oromo people", 'Wolaita Dinich' (potato of the Wolayita people), 'Agew Dinch' (potato of the Agew people) and 'Gurage Dinich' (potato of the Gurage people) depending on the place where *P. edulis* is grown (Mulugeta Taye *et al.,* 2007). We shall hereafter refer to *P. edulis* as Oromo Dinich.

P. edulis (synonym *Coleus edulis*) is a diploid, dicotyledonous plant, with a height of up to 150 cm belonging to the Labiatae (Lamiaceae) family (Mulugeta Taye, 2008). It occurs both as wild and cultivated species. The genus *Plectranthus* has about 32 species in Ethiopia (Hedberg, *et al*, 2006). The cultivated species is grown in the wetter south and south western of Ethiopia whereas the wild species are found throughout the country (PGRC, 1996; IBC, 2007). It also occurs in marshy areas at altitudinal range of 1300-2600 m. In some strains, it produces edible underground potato-like tubers on slender rhizomes (Fig. 1). Oromo dinich (*P. edulis*), like Anchote (*Coccinia abyssinica*) and Coffee (*Coffee arabica*) is believed to have originated in Ethiopia (Dandena Gelmesa, 2010; IBC, 2005).

Yeshitila Mekbib (2007) reported that earlier farmers of Welayta 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 farmers.

Figure 1. Tubers of *P. edulis* locals from Holeta (A) and Welayta (B). Bars represent 2 cm.

Among those, the three local cultivars, namely chenkuwa, lofuwa and unnuka were found to be common and widely distributed (dominant) in all the growing areas of Welayta and were evenly distributed. The dominance of the three major local cultivars was found to be associated with the specific qualities attached to each cultivar. For instance, the chenkuwa cultivar was highly valued by farmers for its good taste and long shelf life. Farmers also stated that this cultivar is drought tolerant and provide some harvest even in bad seasons. On the other hand, cultivars such as lofuwa and unnuka are early maturing types and give relatively better yield (Yeshitila Mekbib, 2007).

2.1.1. *P. edulis* cultivation in Ethiopia

P. edulis is one of the plants propagated vegetatively using the edible parts, i.e. the tuber pieces, whole tubers, stem cuttings and sprout cuttings. The tuber pieces, which are most frequently used (Mulugeta Taye, 2008) are principally obtained from the previous crop or market (Yeshitila Mekbib, 2007). *P. edulis* needs a relatively fertile land in contrast to other root crop species and its cultivation technique is laborious. The main inputs required for production of *P. edulis* are seed tubers, farmyard manure and labor.

Most farmers used at least a quarter of their tubers as a source of seed tubers for the next planting season and hence shortage of seed tuber was noticed as one of the major reasons for the decline in the production of this plant (Mulugeta Taye, 2008). Farmers prefer to plant the tubers in pieces than the whole tuber. Depending on the size of the tuber, they divide a single tuber into 3 to 5 pieces and plant all the tuber pieces in one hill. According to the farmers, using tuber pieces will result in more number of primary stems and progenies than using the whole tuber. This practice would also enable them to get more yields.

P. edulis is a manure demanding crop and the application of organic manure provides a favorable growing environment for *P. edulis* by improving the structure and water holding capacity of the soil. Weeding is done at all stages of crop development and hand-weeding is the common practice employed. Farmers also practice pruning once or twice shortly after the second cultivation in order to have as many progenies as possible and retard the excessive vertical growth of the plant. Once pruning is practiced more progenies will start to emerge. Earthing up takes place 1 to 3 times. The earthing up is mainly carried out so as to cover the runner like structures that emerged at the base of the stem which finally end up producing tubers. Farmers also practice crop rotations and they reported that the yields of crops that immediately follow *P. edulis* in the rotation are benefited (Mulugeta Taye, 2008).

The crop is harvested 6-8 months after planting depending on the type of cultivar. Farmers indicated that the number of progeny tubers from well managed farms varied from 80 to 120 per hole and from poorly managed farms varied up to 20 per hole. The fresh tuber yield per hole varied from 500 to 1000 g in weight. Tubers for consumption were stored in situ in the ground, i.e. in the place where the crop was planted, for a maximum period of 5 months, but usually shorter (Mulugeta Taye, 2008).

2.2. Significance and use of *P. edulis*: Its role in food security

Root and tuber crops, including aroids, are main stays for millions of people and occupy an important position in world agriculture. Like the major crop species, potato, cassava, taro and yams, there are about 100 root and tuber species of significance for agricultural or medicinal purposes. Most of these may be important only locally, but play a significant role in the subsistence economies and crop diversification (MAFF Research Council, 1994). For instance, the yield of *P. edulis* is comparable to that of other root and tuber crops. Weyessa Gardew *et al.* (2009) reported *P. edulis* has comparable yield to potato with yield range of 536.9 to 1008.9 gram per plant.

Indigenous vegetables and tubers have rescued thousands of hungry Ethiopians during famine period and it is a bridge during periods of grain shortage, crucial to food security (Dandena Gelmesa, 2010). For instance, *P. edulis* has been grown and used as a major source of food in many parts of Ethiopia and is liked as a tasty source of carbohydrates (Mulugeta Taye, 2008). They are species that have perhaps not been fully exploited or whose potential has not been fully taken advantage of possibly because their cultivation is restricted and their use localized. Some of these species have a nutritive value and other characteristics that make them very promising candidates for more extensive use. These crops are very important in world food production and for industry, fodder, medicines and in subsistence agriculture. Livington potato (*P. esculetus*) is tuber crop which contributes significantly to nutritional security in some rural communities of Nigeria (Okereke, 2012). *P. edulis* is one of such crops which can contribute high energy carbohydrates comparable to sweet potato and cassava (Mulugeta Taye, 2008).

2.2.1. Nutritive value

Table 1 shows that cooked of *P. edulis* has high contents of food energy, fat, carbohydrate and minerals like phosphorous and calcium than those of *Solanum tuberosum*. Moreover, protein, minerals like phosphorous and iron, and vitamin (thiamin) content of cooked *P. edulis* is higher than that of *Ipomoea batatas.*

Table 1. Nutritional content of *Plectranthus edulis, Solanum tuberosum* and *Ipomoea batatas* (all values per 100 g edible portion)

Composition	*P. edulis*		*S. tuberosum*		*I. batatas*	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
Food energy (Calories)	69.00	100.60	103.7	89.7	136.00	134.20
Moisture (%)	81.90	73.80	73.10	76.80	67.40	65.60
Nitrogen (gm)	0.30	0.24	0.30	0.26	0.30	0.13
Protein (gm)	1.50	1.00	1.30	1.10	1.30	0.50
Fat (gm)	0.20	0.20	0.10	0.10	2.00	0.20
Carbohydrate (gm)	15.30	23.70	24.4	21.10	28.20	32.60
Fiber (gm)	0.70	1.00	1.40	0.90	1.10	1.50
Ash (gm)	1.10	1.30	1.10	0.90	1.10	1.10
Calcium (mg)	29.00	19.00	14.00	9.00	52.00	53.00
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.

Tropical root and tuber crops (cassava, yam and cocoyam) are major staples in large parts of humid and sub-humid sub-Saharan Africa (SSA). For example, SSA produces over 50% of the cassava (*Manihot esculenta* Crantz) and over 90% of the yam (*Dioscorea spp.*) in the world (IITA, 2008). There are several indigenous cultivated or semi-cultivated root and tuber crops in Ethiopia. These crops have an important place in the diet of the population (IBC, 2007). Similarly, *P. edulis*, one of the crops originated from Ethiopia is inexpensive and accessible source of essential nutrients to a country like Ethiopia where the people experience malnutrition problems (Dandena Gelmesa, 2010).

At present, there is a huge interest from the farmers, government and nongovernmental organizations to maintain the crop and increase its production, firstly because it contributes significantly to household food security (Yeshitila Mekbib, 2007), secondly because it is also seen as a traditional food (Mulugeta Taye, 2008).

2.3. Major production constraints in *P. edulis*

2.3.1. Traditional propagation

In recent years, acreage and production of *P. edulis* have declined considerably. One of the major reasons to this decline in production is shortage of seed tuber (Mulugeta Taye, 2008). Most farmers used at least quarter of their plants (product from the previous planting) as a source of seed tuber for the next planting season or they should invest high price to purchase seed tubers (Mulugeta Taye, 2008).

P. edulis, like other important crop plants is propagated vegetatively and grown as clones. Although there is no study regarding disease type and degree of prevalence so far, farmers do report attacks by diseases and insect pests in *P. edulis* (Mulugeta Taye, 2008). Crop plants,

especially vegetatively propagated varieties are generally infected with pathogens (Brown and Thorpe, 1995). *P. edulis,* as tuber crop, being propagated vegetatively, is inherently more susceptible to the maintenance, increase, and dissemination of both systemic and nonsystemic diseases than are the sexually reproduced crops using true seed as planting material. The systemic diseases-viruses, viroids, and mycoplasma pathogens, as well as several bacteria are the most devastating in terms of yield loss for the root and tuber crops (Bryan, 1983).

Despite its economic importance, *P. edulis* has not been genetically improved and usually propagated vegetatively. Suitable methods for conventional vegetative propagation of plants have been developed over many centuries. Research to improve macropropagation methods continues, but has lost some impetus in recent years with the continued extension of tissue culture for plant multiplication (George and Debergh, 2008).

Ostroshy (2006), on the other hand, shows the disadvantages of using the conventional seed potato tubers for multiplication like low rate of multiplication, has high risk of catching various disease (fungal, viral and bacterial disease) and different pests. In this case micropropagation can offer an alternative solution to this problem through the efficient cloning of selected high-yielding plants.

2.3.2. Impact of disease and pest on the quality and quantity of products

Crop plants, especially vegetatively propagated varieties, are generally infected with pathogens (Brown and Thorpe, 1995). Pathogen attack does not always lead to death of the plant. Many viruses may not even show visible symptoms. However, the presence of viruses in plants can reduce the yield and/or quality of crops (Bhojwani and Razdan, 1996). Similarly according to

the study by Mulugeta Taye (2008) majority of farmers in one *P. edulis* growing region mentioned that diseases reduced the number and/or size of the progeny tubers.

Crop losses are caused in several ways. The first and most important is by yield reduction, in which fewer and smaller roots or tubers are produced. As these losses occur below the ground, they are not noticeable until harvest. This is particularly true for the virus diseases and nematodes (Bryan, 1983). For instance, viruses rapidly accumulate in sweet potato through adventitious propagation leading to detectable levels of cultivar decline of 'Beauregard' sweet potatoes in as few as five generations (Bryan *et al.,* 2003).

2.4. Tissue culture tools applicable for mass production of disease free *P. edulis*

Tissue culture refers to the growth and maintenance of a plant in nutrient medium *in vitro* (Harisha, 2007). It allows the rapid clonal propagation of a large number of plantlets over a short period (Lizarraga *et al.,* 1992). Plant tissue culture begins from the concept of totipotency; each plant cell has the ability to divide and grow into a complete plant, similar to its parent, if the suitable conditions of nutrition, light and temperature are provided (Loyola-Vargas and Vazquez-Flota, 2006). It is the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro*. It is an important tool in both basic and applied studies as well as in commercial application (Loyola-Vargas and Vazquez-Flota, 2006).

It is possible to achieve diverse objectives through the tissue culture method of plant propagation. It is most often sought as an alternative in the propagation of cultivars when conventional methods permit only slow increases in clonal plants, e.g. the orchids. Tissue culture propagation is particularly helpful when used in conjunction with plant breeding

programs. It enables the timely increase and hastens the availability of new varieties (Murashige, 1974).

Even with cultivars that are propagated readily through cuttings, divisions, and other conventional asexual techniques, the tissue culture method can be utilized to enhance substantially the rate of multiplication. A millionfold increase per year in the rate of clonal multiplication over conventional methods is not unrealistic. Hence, with plants such as chrysanthemums and others that are already propagated quite rapidly by stem cuttings, tissue culture may be a desirable aid in meeting special needs. In commercial nurseries, tissue cultures can also be used to minimize the growing space usually provided for the maintenance of stock plants. Furthermore, when properly executed, the method can be used in the reproduction and maintenance of relatively disease-free plants (Murashige, 1974).

However, the usefulness of this technology goes beyond academic laboratories. Massive propagation of plants represents an economically rewarding enterprise as a number of companies report significant profits every year. During the past three decades, several companies were established in different countries around the world, and good amount of money was invested in this respect (Omar and Aouine, 2007).

Choice of propagation method is not only depend on the plant species but also on the development of proven techniques, relative costs and agronomic objectives. For example vegetatively reproducing plants can be produced on a large scale by micropropagation (George and Debergh, 2008).

Indexing tests suggest that many cultivars are wholly infected with one or more viruses, and healthy clones must be obtained by eliminating the viruses. Meristem culture is one of the

major techniques for elimination of virus from plant (Hollings, 1965). The technique of meristem culture may be exploited in situations where the donor plant is infected with viral, bacterial, or fungal pathogens, whether or not symptoms of the infection are expressed. The basis of eradication is that the terminal region of the shoot meristem, above the zone of vascular differentiation, is unlikely to contain pathogenic particles.

If a sufficiently small explants can be taken from an infected donor and raised successfully in vitro, then there is a real possibility of the derived culture being pathogen-free (Stepan, 1990). Because most rapid multiplication techniques in root and tuber crops involve the use of aerial portions of the plant, contact with soil and tuber portions is broken and most nonsystemic pathogens and pests can be eliminated (Bryan, 1983). Recent progress using thermotherapy and meristem techniques have enabled scientists to remove many of these systemic diseases from planting materials (Stepan, 1990).

In general, this is a reliable strategy but there are examples where, either because the apical explant was too large or because the pathogen was highly invasive of the apical tissue, this approach has not been successful. In these cases it is good to use two or more techniques together like the plants can be heat treated or exposed to antibiotics *in vitro* then accompanied by meristem culture (Hollings, 1965).

2.4.1. Micropropagation of root crops

There are several different ways to clone or propagate plants vegetatively: micropropagation is a method of propagating plants by culturing very small parts of it called explants (Jha and Biswajit, 2005). It is the true-to-type propagation of selected genotype using *in vitro* culture techniques. Micropropagation derived its name from the miniature shoots initially produced from

this method of plant propagation. This technique provides a rapid and reliable system for production of a large number of genetically uniform disease-free plantlets. The frequency of genetically aberrant plants can be reduced considerably, even avoided entirely, if the plant multiplication can be achieved through an enhancement of axillary shoot formation (Murashige, 1974). It is one of the important contributions of plant tissue culture to commercial plant propagation and has vast significance (Jha and Biswajit, 2005).

Ethiopian government is wishing to motivate farmers to use improved seed, improved varieties, and modern techniques. To ensure that the government requires quality seed and related inputs those are available to the farmers in large volume at the right time.

P. edulis is propagated either from storage roots or from a stem cutting in conventional propagation. However, these methods may be fruitless to maintain disease free plants, mainly due to viral disease and lower multiplication rate. One of the most important features of micropropagation is that it can be a very efficient method of plant propagation. Micropropagation *in vitro* based on meristem culture believed to have even greater potential (Puonti-Kaerlas, 1998).

The potato is a classic example, in which the two or threefold increase in yield in the last 30 years has been very largely due to improved health of seed potato stocks (Hollings, 1965). Increased use of rapid multiplication techniques is enabling scientists to produce large amounts of 'pathogen-free' material (Bryan, 1983). Sweet potato micropropagation, accompanied with an effective seed certification program, ensures that high quality planting stock is available for growers (Bryan *et al.,* 2003). The use of this *in vitro* produced propagation material (seed) can be

possible way to overcome shortage of seed tubers which was noticed by Mulugeta Taye (2008) as one alternative strategy.

A commonly held concern in micropropagation was that tissue culture might reduce the quality of the propagated plants. Many researchers found later that this fear was unnecessary. Sweet potato micropropagation, accompanied with an effective seed certification program, ensures that high quality planting material is available for growers (Bryan *et al.,* 2003). For instance, in various parts of America; North Carolina, Louisiana and California, the use of micropropagation has been found to greatly improve sweet potato yield and storage root quality (Dangler, 1994). Tissue cultured sweet potatoes are used for commercial production in countries such as Kenya, South Africa, Zimbabwe, Egypt, Uganda, and Nigeria where specific biosafety guidelines exist on biotechnology (Mutandwa, 2008).

Along with the successes there are several limiting factors to use micropropagation. The cost of the labour needed to transfer tissue repeatedly between vessels and the need for asepsis can account for up to 70% of the production costs of micropropagation. Problems of vitrification, acclimatization and contamination can cause great losses in a tissue culture laboratory. Genetic variations in cultured lines, such as polyploidy, aneuploidy and mutations, have been reported in several systems and resulted in the loss of desirable economic traits in the tissue-cultured products (Jha and Biswajit, 2005).

Phenol oxidation is a common problem in tissue culture when plant organs are cut for transference to media. It can lead to undesirable wound responses. Tissues containing relatively high concentrations of phenolic compounds are difficult to culture. Polyphenolases stimulated by tissue injury will oxidize these phenolic substances to growth-inhibiting, dark-colored

compounds (Dodds and Roberts, 1985). Nowadays, a promising result was found through the use of specific antioxidant pretreatments such as the use of ascorbic acid, citric acid and L-cysteine hydrochloride for seed embryos and meristems of oak. Soaking explants in antioxidants was more beneficial for the meristems than for the seed embryos (Haapala, 2004).

In acclimatization, very high temperatures, light and low humidity separately or together caused low survival. It was concluded that any temperature above 55°C caused death, causing cassava to wilt before dying. The combination of high light intensity (above 500-800 μmol m^{-2} s^{-1}) and temperatures above 40°C was also lethal to plants that showed bleached symptoms due to a photo-oxidation effect. In addition to the combined effects of light and temperature, the relative humidity was also shown to play an important and decisive role in plant survival, when critical temperatures and high light are present. High relative humidity (70-90 %) although essential during the first week after transplant, was detrimental for plant survival if maintained until the fourth week after transplant, causing yellowing leaves, the plants to rot and fungal infestations due to excessive humidity (Koehorst-van *et al.,* 2012). However, as species differ greatly in their requirements there is no universal acclimatizing protocol (Seelye *et al.,* 2003). In most cases, successful acclimatization requires two weeks of high relative humidity, temperature below 55^{o}C and relatively low light provided by light shade (Koehorst-van *et al.,* 2012).

A cell culture contaminant is some element in the culture system that is undesirable because its possible adverse effects on either the system or its use. These elements are types: chemical contaminants like metal ions, plasticizers, free radicals deposits on different materials, residues and impurities. These contaminants may come from the media, water endotoxins, storage vessels and fluorescent light we sued. The other type is biological contaminants like bacteria, molds and yeast those that are usually easy to detect and viruses, protozoa, insects, mycoplasmas and other

cell lines those that are more difficult to detect, and as a result potentially more serious culture problems (Ryan, 2002).

The most obvious consequence of cell culture contamination is the loss of time, money (for cells, culture vessels, media and sera) and effort spent developing cultures and setting up experiments. However, the less obvious consequences are often more serious. First there are the adverse effects on cultures suffering from undetected chemical or biological contaminants. Worse yet are the potentially inaccurate or erroneous results obtained by unknowingly working with these cryptically contaminated cultures. For some researchers the most serious consequence of contamination is suffering the embarrassment and damage to their reputation that results when they notify collaborators or journals that their experimental results are faulty and must be retracted due to contaminants in their cultures (Ryan, 2002).

2.4.2. Micropropagation from meristem culture

Meristem culture is the excision of the organized apex of the shoot from a selected donor plant for subsequent *in vitro* culture. The excised meristem is typically small (often less than 1 mm in length) and removed by sterile dissection under the microscope. It comprises the apical dome and a limited number of the youngest leaf primordia (Jha and Biswajit, 2005), and excludes any differentiated provascular or vascular tissues (Stepan, 1990).

Meristem cultures are used for virus and bacteria elimination. Meristem cultures are initiated from much smaller explants and a single plantlet is usually produced from each (George and Debergh, 2008). The explant being extremely small, the rates of survival and development *in vitro* are distinctly poor. However, the probability of isolating a clean tissue, i.e. an explant free from microorganisms, is inversely related to its size (Murashige, 1974). The isolation of the

meristematic zone under aseptic conditions and its culture in an adequate nutritive medium allow plantlet development with a differentiation pattern similar to that of a normal plant (Lizarraga *et al.,* 1992).

A major advantage of working with meristems is the high probability of excluding pathogenic organisms, present in the donor plant, from cultures (Honda *et al.,* 2001; Stepan, 1990). Meristem culture alone has enabled virus-free clones to be obtained with some plants (Hollings, 1965). Several reasons such as absence of plasmodesmata in the meristematic domes, faster cell division, competition between synthesis of nucleoproteins for cellular division and viral replication and presence of inhibitor substances make meristem useful source to obtain virus free plantlets. With viruses so far tested, different workers agree that the virus concentration diminishes sharply in the terminal 5 mm or so of the shoot apex (Hollings, 1965).

Another advantage is the genetic stability inherent in the technique. Plantlet production is from an already differentiated apical meristem and propagation from adventitious meristems can be avoided (Loyola-Vargas and Vazquez-Flota, 2006; Stepan, 1990). Shoot development directly from the meristem avoids callus tissue formation and adventitious organogenesis, ensuring that genetic instability and somaclonal variation are minimized. Commonly, the selection of the explant that is used to introduce the plant into culture is made on the basis of the genetic stability and suitability for efficient propagation of the tissue *in vitro*.

Adesoye *et al.* (2012) found that shoot induction from shoot tip developed earlier and responded better than those from meristem. However, the fact remains that meristem explants have the advantage of producing plantlets that are virus free which, however, cannot be guaranteed for plantlets produced from shoot tip or any other part of the parent plant. Thus, uniform and clean

seed derived from mericlones may be economically feasible for growers compared to conventional propagation (Alam *et al.,* 2010). The culture conditions are controlled to allow only organized outgrowth of the apex directly into a shoot, without the formation of any adventitious organs, ensuring the genetic stability of the regenerated plants.

Meristem and node cultures are the most reliable for micropropagation to produce true-to-type plants (Honda *et al.,* 2001). Rout *et al.* (1998) utilized RAPD markers for the assessment of genetic stability of clonal materials of Ginger (*Zingiber officinales*). In their work no variation was detected within the micropropagated plants and they were similar to the field grown control plants. By the use of RAPD and ISSR markers, Sreedhar *et al.* (2007) also concluded that the micropropagation protocol that have been used for *in vitro* proliferation of vanilla plantlets for the last 10 years might be applicable for the production of clonal plants over a considerable period of time.

Moreover this technique adds a high propagation rate to the original meristem culture technique, and together the techniques form the basis of micropropagation, if the expanded internodes subdivided in to new segments and cultured into fresh medium (Stepan, 1990).

It is possible, however, that callus tissue may develop on certain portions of the growing explant, particularly at the surface damaged by excision. The only acceptable situation under such circumstances is that the callus be sufficiently slow growing and localized to be readily identified, and that it and any organized development from it be excised at the first available opportunity (Honda *et al.,* 2001).

In selecting stock plants for tissue culture, the main question is not whether they are infected on the surface with pest or microorganisms that, in principle, can be eliminated by surface

sterilization of the explant, but whether there are inter- or intra-cellular endophytes that may enter the cultures to cause contamination. Where stock plants are showing systemic disease symptoms, they should preferably be rejected and disease escapes sought. If the latter are not available, then meristem culture should be used to introduce the plants into culture. There is, however, the risk that phytopathogens (as opposed to viropathogens) may be latent in the plant and in the established cultures and so the stock plants must be formally indexed for specified pathogens in the case of crops for certification (Loyola-Vargas and Vazquez-Flota, 2006).

2.4.3. Effect of growth regulators on meristem culture and micropropagation

Plant growth regulators (PGRs) are signal molecules produced within the plant at extremely low concentrations (Sunilkumar, 2005). Growth regulators or plant hormones, such as auxins, gibberellins, abscisic acid, cytokinins, and ethylene, are known to influence various stages of growth in the whole plant. Of these, only auxins and cytokinins are routinely incorporated in to plant culture medium.

They are most critical organic components of plant propagation media (Murashige, 1974). The most commonly used auxins are 2,4-dichlorophenoxyacetic acid (2,4-D), indole 3- acetic acid (IAA), and l-naphthaleneacetic acid (NAA) (Kalilian, and Faramarz, 1990). Indole-3-butyric acid (IBA) is another auxin which is used less frequently in tissue culture. Most frequently used cytokinins 2iP, BA and Kinetin are readily available on the commercial market. The cytokinin 2iP is the most active of the three; it is, however, the most expensive. Kinetin and BA are nearly equivalent in effectiveness, perhaps BA being slightly more (Murashige, 1974). Gibberellins, while stimulating growth of organs, generally repress organ initiation processes.

The choice of type and concentration of hormones are the most important considerations in preparation of the medium (Dodds and Roberts, 1985). By careful manipulations of the relative concentrations of auxins and cytokinins, it is possible to favor either undifferentiated growth or organogenesis (root or shoot formation) (Kalilian and Faramarz, 1990). Both substances are necessary for tissue growth, the pattern of organogenesis is determined by their relative concentrations in the nutrient medium. A relatively high concentration of auxin favors root initiation, while repressing shoot formation. In contrast, relatively high concentrations of cytokinin induce shoot initiation and suppress rooting (Murashige, 1974).

GA_3 and BAP significantly affect *in vitro* multiplication of potato. GA_3 can significantly increase the shoot length during *in vitro* rapid multiplication at higher concentrations. BAP can bring significant improvement in *in vitro* multiple shoot induction by increasing the number of shoots when used at moderate concentrations (Rabbani *et al*., 2001). Whereas an increase in BAP concentration beyond the optimal level led decrease in length and aggregations of shoot buds in *Mucuna pruriens* (Hanna Beksissa, 2006).

3. Objectives

3.1. General objective

- To develop micropropagation protocol for *Plectranthus edulis* using meristem as explant.

3.2. Specific objectives

- To induce shoots from meristem of *P. edulis;*
- To determine the optimum plant growth regulators concentration for shoot multiplication;
- To determine optimum concentration of auxins for rooting of microshoots;
- To determine optimal combination of plant growth regulator;
- To acclimatize *in vitro* grown *P. edulis* plantlets at glasshouse.

4. Material and Methods

4.1. Plant material

Seed tubers of *P. edulis* of Holeta (local market) and Welayta (from farmers) were planted in the glasshouse at College of Natural Sciences, Addis Ababa University. About 1.5-2.0 cm long shoots were collected from two-month-old glasshouse grown mother plants (Fig. 2).

Figure 2. Mother plant for culture initiation of *P. edulis*. Seed tubers of Holeta (A), and Welayta (B) locals, and seedlings grown in glasshouse from Holeta (C) and Welayta local (D). Bars represent 5 cm.

4.2. Media preparation

4.2.1. MS medium stock preparation

The MS (Murashige and Skoog, 1962) basal medium, four different stock solutions were prepared as recommended by Murashige and Skoog (see Appendix 1).

4.2.2. Growth regulators stock preparation

Different growth regulators; 6-benzyl aminopurine (BAP), Gibberrelic acid (GA_3), α-naphthalene acetic acid (NAA), Indol acetic acid (IAA) and indol-3-butyric acid (IBA) were used for this study. All growth regulator stock solutions were prepared by weighing and dissolving the powder in double distilled water at the concentration of 1.0 mg/ml. To begin the dissolving process, 3-4 drops of 1M NaOH, HCl or 94% ethanol were added based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin and ethanol for gibbereline were used). Then, the volume was adjusted by adding double distilled water. Finally, growth regulators stock solutions were stored in a refrigerator at a temperature of 4^0C for immediate use.

4.2.3. Culture medium preparation

Culture medium were prepared by taking proper amount of MS stock solution (50 ml/l macro, 5 ml/l micro including iron-EDTA and 5 ml/l vitamin) and different concentrations and combinations of plant growth regulators. Sucrose (30 g) were weighed and dissolved in double distilled water in all cases.

Medium for meristem culture were prepared using MS medium supplemented with different concentrations of BAP (0.1, 0.5, 1.0, 2.0 or 5.0 mg/l) in combination with 0.1 mg/l NAA and 1.0 mg/l GA_3. After all these constituents including sucrose dissolved and thoroughly mixed in 1 L

volumetric flask, and transferred to two liter beaker or bottle and 8 g agar was added after the pH was adjusted to 5.8 using 1M HCI and/or 1M NaOH and autoclaved at 121^{o}C for 15 minutes. Then 20 ml medium was poured into sterile Petri-dishes and then media were kept until cooled to room temperature.

Shoot multiplication medium was composed of MS medium containing different concentrations of BAP (0.1, 0.5 or 1.0 mg/l) or Kinetin (0.5, 1.0, 2.0 or 3.0 mg/l) in combination with NAA (0.05 or 0.1 mg/l). In media preparation, medium was melted with microwave oven and 50 ml of it were dispensed in to each Magenta GA-7 vessel before it was autoclaved at 121^{o}C for 15 minutes.

For rooting, full and half-strength (1/2) MS medium containing 1.0 mg/l of IAA, IBA or NAA were prepared and poured into sterile test tubes.

4.3. Sterilization of explants

Shoot explants were washed twice with tap water and detergent, and rinsed with double distilled water twice. Then, surface sterilized using 70 % alcohol for 30 seconds and rinsed three times with sterile double distilled water followed by sterilization by 0.5 % Sodium hypochlorite solution (NaClO) containing three drops of Tween 20 for 10 minutes. After sterilization, the explants were rinsed three times with sterile double distilled water.

4.4. Meristem isolation and shoot induction

The upper leaves from sterilized shoots were removed. Leaves and leaf primordia were removed one by one and shoot apical meristems, consisting of the apical part and one or two leaf primordia, were isolated carefully under dissecting microscope using sterile forceps, scalpels and hypodermic needles. Each meristem with one or two leaf primordia (0.2-0.5 mm) were excised

and cultured immediately on MS medium containing 0.1 mg/l NAA, 1.0 mg/l GA_3 and different concentrations of BAP (0.1, 0.5, 1.0, 2.0 or 5.0 mg/l).

Explants were cultured in 90 mm diameter Petri dishes each containing 20 ml medium. For each treatment a total of 30 explants were used. There were five explants per Petri dish with six replications. Numbers of dead meristem and number of meristems that were induced to shoots were recorded. Cultures were transferred to fresh media every two weeks until shoots were initiated. The cultures were maintained at a temperature of 25 ± 2 ^{0}C under light intensity of 20 µmol m^{-2} s^{-1} and 16 h photoperiod provided by cool-white fluorescent lamp. The experiment was repeated once.

4.5. Shoot multiplication

Initiated shoots were cultured on shoot multiplication medium. Shoot multiplication medium was MS medium containing different concentrations of BAP (0.1, 0.5, or 1.0 mg/l) or Kinetin (0.5, 1.0, 2.0 or 3.0 mg/l) alone or in combination with NAA (0.05 or 0.1 mg/l). Shoots were cultured in magenta GA-7 vessels containing 50 ml medium. For each treatment, a total of 30 explants were used. The cultures were incubated at 25 ^{o}C ± 2 at light intensity of 20 µmol m^{-2} s^{-1} under 16 h photoperiod provided by cool white fluorescent light.

Number of shoots per shoot explant was recorded every four weeks. The effects of different treatments were quantified on the basis of number of shoot per explants per treatment. The whole experiment was repeated once. Number multiple shoots per explants and length of shoots were also recorded while sub-culturing. The two consecutive sub-culturing and mean values was used for analysis of variance (ANOVA) and mean value for each treatment were compared by least significance difference (LSD).

4.6. Rooting

Rooting was attempted both *in vitro* and *ex vitro*. *In vitro* rooting was carried out in two ways: Firstly, multiplied shoots were cultured on both full and half strength MS medium containing 1.0 mg/l NAA, IAA or IBA. Secondly, shoots were briefly immersed into 5.0 mg/l IBA for five minutes before transferring into plant growth regulator free MS medium. Shoots were incubated for a week in darkness and then transferred to a 16-h photoperiod, produced from cool white fluorescent lamps for three weeks. In *ex vitro* rooting, shoots were transferred to small pots filled with soil, compost and sand in 2:1:1 ratio, respectively after briefly immersed into 5.0 mg/l IBA for five minutes and covered with moistened polyethylene bags. The polyethylene bags were removed from the pots after a week under glasshouse condition. Rooting was evaluated in terms of rooting percentage, root number, the mean root length after one month of culturing.

4.7. Acclimatization

After four weeks in rooting medium, *in vitro* rooted shoots were washed thoroughly to remove residual medium and transferred to plastic pots containing a mixture of soil, compost and sand in a ratio of 2:1:1, respectively. The washing was done in such a way that roots were damaged to a minimum level. Each pot was covered with polyethylene bag and kept in glasshouse. The polyethylene bags were removed after a week. The numbers of surviving plants in the glasshouse were recorded after a month.

4.8. Statistical analysis

Statistical analysis of quantitative data was carried out by SPSS computer software of version 16. A difference at probability level of $p \leq 0.05$ was considered significant for analyses. 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. Shoot induction from meristem

The use of solid MS medium containing BAP, GA_3 and NAA resulted in the development of shoots from meristem without callus formation. As it was shown in Fig. 3 the earlier sign of growth from meristem was noticeable within 7 days after culture of the explants on MS medium containing 1.0 mg/l GA_3, 0.1 mg/l NAA and different concentration of BAP (0.1, 0.5, 1.0, 2.0 or 5.0 mg/l BAP). Growth was continued to shoot development, resulting in primary shoot establishment. Meristems were differentiated into either single or multiple shoots.

Table 2. Effects of different concentrations of BAP combined with GA_3 (1.0 mg/l) and NAA (0.1 mg/l) on shoot induction from meristem explants of *P. edulis* after 6 weeks of culture

Plant Growth Regulators			Shoot induction			
BAP (mg/l)	GA_3 (mg/l)	NAA (mg/l)	Holeta local		Welayta local	
			No.	%	No.	%
0.0	0.0	0.0	6	10	8	13
0.1	1.0	0.1	44	73	36	60
0.5	1.0	0.1	32	53	34	57
1.0	1.0	0.1	43	72	39	65
2.0	1.0	0.1	32	53	35	58
5.0	1.0	0.1	35	58	31	52

ANOVA result showed that cytokinin concentration significantly affected percentage of shoot induction ($P \leq 0.05$). However, there is no statistically significant difference between plants of the two collection areas in percentage of shoot induction. The highest percent shoot induction

was achieved by two of the growth regulator combination (see Table 2). These are 0.1 mg/l and 1.0 mg/l BAP in combination with 1.0 mg/l GA_3 and 0.1 mg/l NAA.

In this study, the least percent shoot induction was observed in growth regulator free MS medium (10 % from Holeta and 13 % from Welayta local), but later growth slowed and stay stunted.

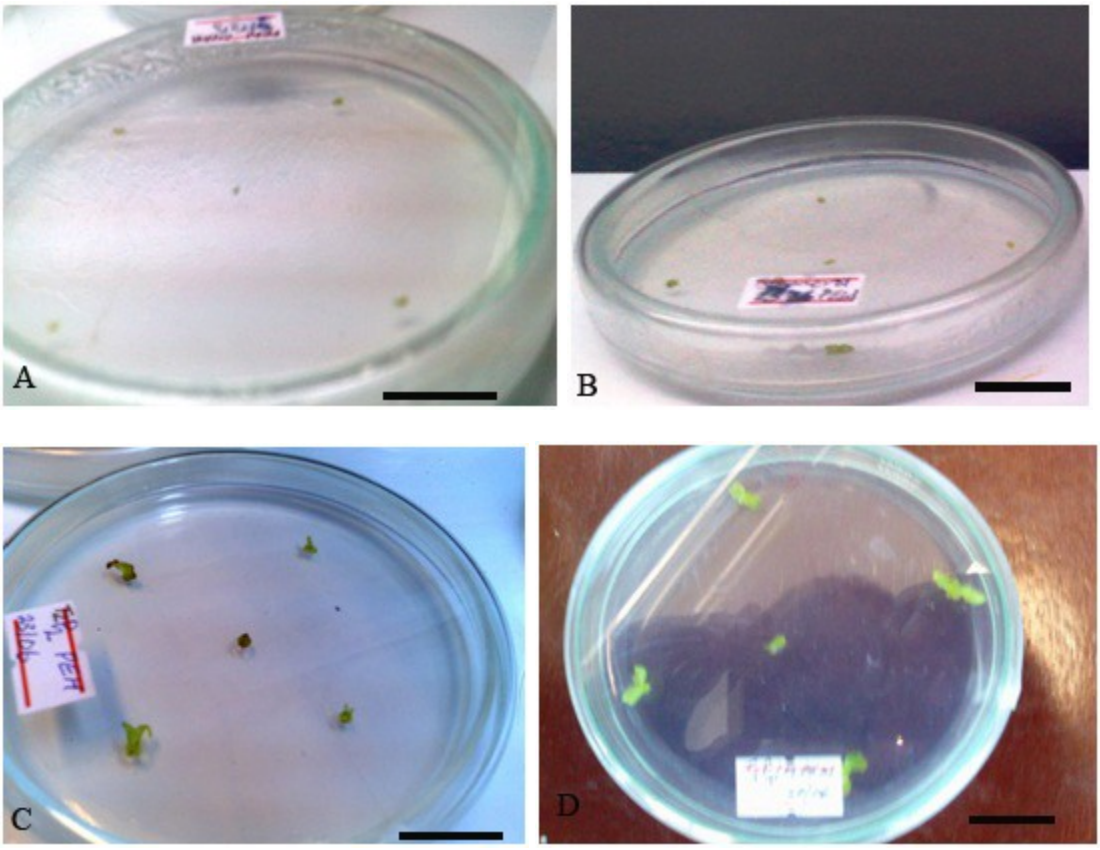


Figure 3. Response of meristem culture of the two groups of *P. edulis* on 1.0 mg/l BAP in combination with 1.0 mg/l GA_3 and 0.1 mg/l NAA. (A) Holeta and (B) Welayta after a week; (C) Holeta and (D) Welayta after 20 days. Bars represent 2 cm.

Although there was no significant difference among treatments, percentage of shoot induction in these treatments (1.0 mg/l GA_3, 0.1 mg/l NAA and 0.1 to 5.0 mg/l BAP) was ranged from 52 % to 73 %.

Although in all concentration of BAP shoot induction was triggered, more profuse shoot induction was observed at 1.0 mg/l BAP with full potential of giving rise to best regenerative shoots. Shoots developed have better quality in appearance than those developed on MS medium containing BAP above 2.0 mg/l. The shoots were relatively deep green colored independently surviving shoots when subcultured. The percentage of shoot induction was 72 % from Holeta and 65 % from Welayta local on this medium. However, combination of 0.1 mg/l BAP with 1.0 mg/l GA_3 and 0.1 mg/l NAA resulted in highest percentage (73 %) shoot induction.

Although meristems cultured on medium containing 0.1 mg/l NAA, 1.0 mg/l GA_3 and 0.1 mg/l BAP had highest percentage of shoot induction (73 % from Holeta), the plant produced poor quality (whitish like vitrified plant) with few number of shoots compared to plants cultured on 1.0 mg/l BAP. It gave rise to small number of shoots for subculture. Shoots grown in this medium had longer shoots than others.

At higher concentrations of BAP (2.0 or 5.0 mg/l) combined with 0.1 mg/l NAA and 1.0 mg/l GA_3 produced stunted adventitious shoots (Fig. 4). Such shoots grown in relatively higher concentration of cytokinin induced callus like swelling at the base of the shoots. Profuse callus like swelling occurred at the base of most of the meristems grown in such kind of medium. Attempt to induce shoot regeneration from such shoots were less successful.

Figure 4. Shoot induction from meristem culture of Holeta and Welayta locals of *P. edulis* respectively using various concentration of BAP combined with 1.0 mg/l GA_3 and 0.1 mg/l NAA. (A, B) shoots treated with 0.1 mg/l BAP; (C, D) shoots treated with 1.0 mg/l BAP; (E, F) using relatively higher cytokinin (5.0 mg/l BAP). Bars represent 2 cm.

5.2. Shoot multiplication

Shoot number was highly influenced by concentration and type of the growth regulators. Different combination of BAP, KIN and NAA resulted in different response. Among them, 1.0 mg/l BAP in combination with 0.05 mg/l NAA in the media could induce a mean of 5.8 and 4.9 shoots from Holeta and Welayta local per explants, respectively. Among different concentrations and combinations for number of shoot, best performance was obtained on MS medium supplemented with 1.0 mg/l KIN and 0.1 mg/l NAA for Holeta which was mean of 7.2 shoots and 3.0 mg/l KIN and 0.05 mg/l NAA for Welayta which was 6.2 shoots per explant.

However, lower concentration of cytokinin (0.1 mg/l BAP) alone or in combination with 0.05 mg/l NAA could only induce a low percentage of multiple shoots and a few number shoots per explants compared to cultures with moderate or higher concentration of cytokinin.

Generally number of shoots per explant was lower on media containing BAP than those containing Kinetin. On medium containing BAP alone (0.1-1.0 mg/l) shoot elongation was better (see Table 3).

Table 3. Percentage of shoots producing multiple shoots, number and length of shoots per explants that were cultured on MS medium containing different concentration of BAP, Kinetin and NAA

PGR (mg/l)			Explants with multiple shoots (%)		Number of shoots per explant		Shoot length (cm)	
BAP	Kinetin	NAA	Holeta	Welayta	Holeta	Welayta	Holeta	Welayta
0.0	0.0	0.0	16.6	0	1.2^{a}	1.0^{a}	7.2^{a}	7.3^{a}
0.1	0.0	0.0	73.3	76.7	2.1^{bc}	2.1^{a}	4.0^{bc}	4.4^{bc}
0.5	0.0	0.0	100	96.7	4.5^{defg}	4.1^{bcdef}	2.8^{de}	3.0^{de}
1.0	0.0	0.0	100	100	4.9^{defh}	4.8^{bcdeg}	2.6^{d}	2.5^{a}
0.1	0.0	0.05	78.3	85	2.7^{bci}	3.2^{fhi}	3.3^{ef}	4.1^{bcf}
0.5	0.0	0.05	68.3	95	2.7^{bci}	4.5^{bcde}	3.2^{ef}	3.2^{de}
1.0	0.0	0.05	100	100	5.8^{hj}	4.9^{cdeg}	3.0^{def}	3.0^{de}
0.1	0.0	0.1	75	86.7	3.1^{cik}	3.2^{fhi}	4.7^{gh}	4.3^{bc}
0.5	0.0	0.1	100	100	4.6^{defgh}	4.0^{bdef}	3.3^{f}	3.2^{de}
1.0	0.0	0.1	100	100	4.5^{defg}	5.2^{ceg}	3.3^{f}	3.2^{de}
0.0	0.5	0.0	86.7	85	3.3^{cik}	3.2^{fhi}	4.2^{bc}	4.2^{bcf}
0.0	1.0	0.0	90	100	4.1^{dfgk}	4.7^{bcdeg}	4.3^{bc}	4.2^{bcf}
0.0	2.0	0.0	100	100	4.7^{defgh}	4.7^{bcdeg}	3.9^{b}	3.9^{c}
0.0	3.0	0.0	100	100	4.7^{defgh}	4.7^{bcdeg}	3.8^{b}	3.8^{f}
0.0	0.5	0.05	86.7	93.3	3.5^{gik}	3.7^{bfhi}	4.6^{gh}	4.7^{b}
0.0	1.0	0.05	95	90	4.3^{defg}	4.0^{bdef}	4.6^{gh}	4.2^{bcf}
0.0	2.0	0.05	100	100	4.1^{dfgk}	3.9^{bdfi}	4.1^{bc}	4.3^{bc}
0.0	3.0	0.05	100	100	6.6^{l}	6.2^{j}	4.0^{bc}	4.3^{bc}
0.0	0.5	0.1	100	98.3	5.7^{hj}	5.2^{ceg}	4.5^{cgh}	4.6^{b}
0.0	1.0	0.1	100	93.3	7.2^{l}	5.9^{gj}	2.9^{de}	3.3^{de}
0.0	2.0	0.1	100	96.7	5.3^{efhj}	4.6^{bcdeg}	3.2^{ef}	3.3^{e}
0.0	3.0	0.1	100	100	5.5^{ehj}	5.1^{cdeg}	3.5^{f}	4.7^{b}

Means followed by the same letter within a column were not significantly different at 5 % probability.

After 35 days of culture, most of the responding explants of PGR free medium (83.4 % of Holeta and all explants of Welatya) produced only a single shoot with highest mean shoot length of 7.2 cm and 7.3 cm from Holeta and Welayta respectively (Fig. 5). Average length of shoots depended on the type of locals, type and concentration of the growth regulators (Table 3). Next to shoots cultured on PGR free medium, the longest shoot in Holeta and Welayta local were produced on medium containing 0.1 mg/l BAP + 0.1 mg/l NAA and 0.5 and 3.0 mg/l KIN + 0.05 and 0.1 mg/l NAA combination respectively.

Explants cultured on higher BAP (1.0 mg/l) have significantly lower shoot length compared to explants treated with lower BAP (0.1 mg/l).

Presence of NAA in the medium significantly affected the response promoted by BAP or KIN in multiple shoot culture. Shoots cultured on medium containing 1.0 mg/l BAP combined with 0.05 mg/l NAA produced significantly more number of shoots than shoots cultured on same amount of BAP alone.

Among shoots cultured on PGR free MS medium, 75 % of Holeta and 91.7 % of Welayta local showed simultaneous rooting (see Appendix 3). Moreover, there was frequent spontaneous rooting while shoots were maintained on multiplication medium particularly in KIN combined with NAA. Next to PGR free medium, the highest spontaneous rooting of 30 % from Holeta and 20 % from Welayta were exhibited from treatment containing 1.0 mg/l KIN and 0.1 mg/l NAA. Finally, optimal KIN and NAA concentrations with maximum possible multiple shoots varied with type of explants, being 1.0 and 0.1 mg/l for Holeta and 3.0 and 0.05 mg/l for Welayta local.

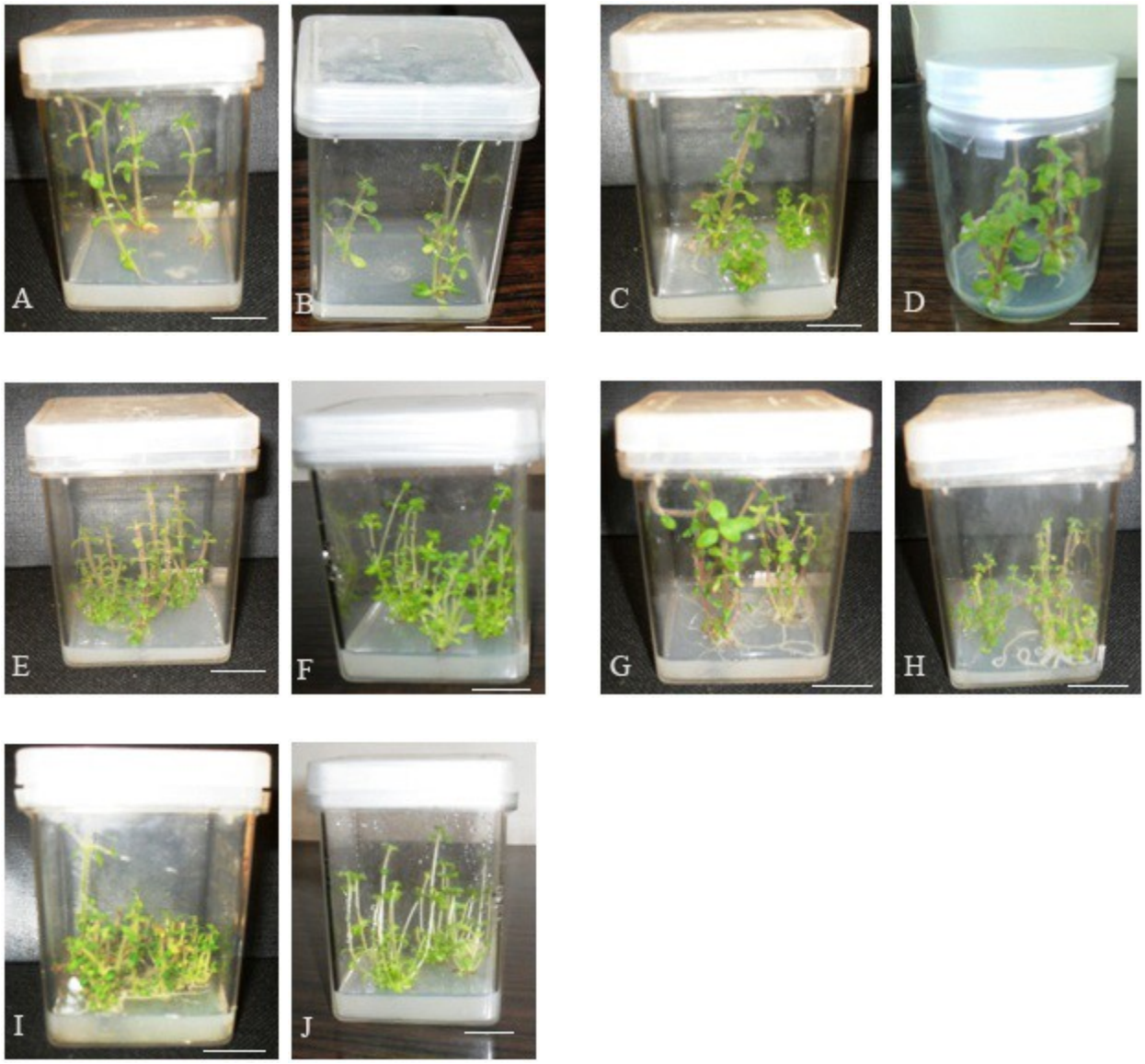


Figure 5. Multiple shoots from Holeta and Welayta area local of *P. edulis* on different shoot multiplication medium. (A, B) PGR free medium; (C, D) 0.1 mg/l BAP alone; (E, F) 1.0 mg/l BAP combined with 0.1 mg/l NAA; (G, H) 0.5 mg/l KIN alone (rooted shoots); (I, J) 3.0 mg/l KIN combined with 0.1 mg/l NAA. Bars represent 2 cm.

5.3. Rooting and acclimatization

Rooting were observed three weeks after culturing the shoots on root induction medium, and most of the shoots developed roots by week four. Overall, Holeta local had a better rooting response as compared to Welayta local in most of the cases (see Table 4).

Table 4. Effect of MS salt strength and auxin on rooting, number of roots per shoot and root length of *P. edulis*. For numbers of rooted shoots see appendix 4.

Treatments		Shoots rooted %		No. of roots/explan		Root length	
MS salt strng.	Auxin (1.0 mg/l)	Holeta	Welayta	Holeta	Welayta	Holeta	Welayta
Full strength	0.0	60	31.7	1.9^{a}	0.84^{a}	1.7^{a}	1.0^{a}
Full strength	NAA	30	35.0	0.59^{b}	0.98^{a}	1.0^{a}	0.9^{a}
Full strength	IAA	10	33.3	0.16^{b}	0.83^{a}	1.5^{a}	1.5^{a}
Full strength	IBA	95	61.7	3.23^{c}	1.61^{b}	1.6^{a}	2.0^{a}
Half strength	IAA	25	10	1.00^{b}	0.12^{c}	1.6^{a}	1.5^{a}
Half strength	IBA	100	76.7	3.12^{c}	2.2^{b}	1.5^{a}	1.1^{a}
*Half strength	0.0	38.3	16.7	0.65^{b}	0.32^{c}	0.9^{a}	0.9^{a}
**Ex vitro*	0.0	100	100	6.17^{d}	5.43^{d}	3.3^{b}	3.3^{b}

Cultures on *Half and **Ex vitro* were grown on regulator free MS medium and potting mix respectively, but the shoots were dip into 5.0 mg/l IBA for 5 minutes before culture into growth regulator free medium and potting mix. Means followed by the same letter within a column were not significantly different at 5 % probability.

5.3.1. Effect of MS salt strength and auxins on root induction

Shoots were rooted best on half strength MS medium. For Holeta locals higher percentage of rooting (100%) was observed when the shoots were cultured on 1.0 mg/l IBA. Out of the two different strength of MS medium, half strength MS medium containing 1.0 mg/l IBA provided

highest rooting percentage (100%) from Holeta and (76.7%) from Welayta local and induced a mean roots of 3.12 from Holeta and 2.2 from Welayta local (Table 4).

Figure 6. Rooted shoots from Holeta and Welayta local of *P. edulis* on different rooting conditions. (A, B) rooted shoots on PGR free medium; (C, D) rooted shoots on MS + 1.0 mg/l IBA; (E, F) rooted shoots on ½ MS + 1.0 mg/l IBA; (G, H) rooted shoots on *ex vitro* condition. Bars represent 2 cm.

5.3.2. *Ex vitro* rooting

In *ex vitro* rooting, out of the shoots transferred to glasshouse condition, shoots produced from Holeta local showed the highest percentage of survival (90 %) compared to Welayta local which

exhibited 76.6 % survival. Compared to acclimatization of *in vitro* rooted plantlets, *ex vitro* rooting was shown least survival percentage of shoots. The maximum rooting percentage in these experiments was 100 %, shown by *ex vitro* rooting by transfer of the shoots from multiplication medium to small pots after shoots were deep in 5.0 mg/l IBA for 5 minutes. Although it showed minimum percentage survival compared to acclimatization, all the survived shoots were rooted and induced a mean of 6.17 and 5.53 roots from Holeta and Welayta locals, respectively and produced the longest mean root length (3.3 cm in both locals). Rooting percentage, number of roots per shoot and root length was significantly different from results of all other treatments for both locals. *Ex vitro* grown shoots had very good morphological appearance (Fig. 6).

After transfer of *in vitro* rooted shoots from culture to the glasshouse, 100 % plantlets of Holeta local and 96.6 % plantlets from Welayta local survived after two weeks of acclimatization and stars to grow healthy (Fig. 7).

Figure 7. Acclimatization of *in vitro* rooted shoots of *P. edulis* in glasshouse; (A,B) Holeta and (C,D) Welayta locals after two weeks. Bars represent 5 cm.

6. Discussion

6.1. Shoot induction from meristem Culture

In this study, the effect of PGR on shoot induction from meristematic explant, shoot multiplication and rooting was investigated. Five different media (treatments) were used to induce shoot from *P. edulis* meristem explants. These media, with various combinations of cytokininns and auxins, triggered direct shoot initiation from the meristem explants. Similar result were obtained for shoot induction from meristem culture by Acedo (2006) although there was difference in the type of the MS medium (liquid medium), plant regeneration from shoot apical of white yam meristems was best enhanced using MS medium containing GA_3, BAP and NAA.

Biological activity of any one substance not only varies with the dosage but depends greatly on the other conditions in which it is placed (Murashige and Skoog, 1962). Combination of Plant growth hormones exerted growth stimulatory effects. Normally, other species like sweet potato and cassava show good response towards plant regeneration in MS medium in the presence of BAP combined with auxins as reported by various authors (Tekalign Wondimu *et al.,* 2012, Acedo, 2006). When used alone, GA_3 did not elicit any growth response while BAP caused shoot formation only (Acedo, 2006). While Dagnino *et al.,* (1991) reported GA_3 has different response to different cultivars of *Ipomoea batatas*, as it has no effect on growth of *Coracao alado* and promotes multiple shoot in Mae de Famillia cultivars.

Shoot development without tissue callusing was effected only when the three growth regulators were combined (Acedo, 2006). Armin *et al.* (2011) found that the best shoot induction response of purple colored sweet potato was obtained on MS medium supplemented with 1.0 mg/l BAP.

Dawit Beyene (2009) also reported the use of 0.5 or 1.0 mg/l BAP in combination with 1.0 mg/l GA_3 and 0.01mg/l NAA gave morphologically good looking shoots in cassava varieties. These findings were consistent with the results of this experiment. In this study, MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l NAA and 1.0 mg/l GA_3 were proved to be the best in terms of quality of shoot and survival in both groups. It is almost in agreement with the work of Adesoye *et al.* (2012) as they obtained best regeneration of sweet potato plant in medium supplemented with 0.5 mg/l BAP and 0.1 mg/l GA_3. Shoots were grown having no callus like structure at the basal end of explants. The quality of shoots and the overall growth response in terms of shoot length was better in this growth regulator combination.

Meristems cultured *in vitro* on shoot culture medium containing only low amount of growth regulators produce one shoot, but they can be induced to form multiple shoots on cytokinin containing medium (Puonti-Kaerlas, 1998). In our study most of meristem induced into multiple shoots directly from meristem.

The shoots developed from higher cytokinin (2.0-5.0 mg/l BAP) treatments were numerous in number, but grown slowly and form big callus like structure at the base of the shoots. They were not effectively regenerated in successive subcultures. Such kinds of shoots were reported by Yasmin *et al.* (2011) in potato as in most of combination of higher concentration of BAP and NAA explants formed shoots and calli at the base except on 0.5 mg/l BAP and 0.5 mg/l NAA which were regenerated having no calli at the basal end of explants.

Usually, no exogenous growth regulators are required if the apical explants (meristem) contain two or more pairs of primordia leaves (Altaf, 2009). But the report on potato proliferation by Yasmin *et al.* (2011) shows that growth of explants is slow in such hormones free, cost effective

media. This result is in agreement with this study, the least shoot induction percentage was observed in growth regulator free medium (about 10 % from Holeta and 13 % from Welayta). The growth slowed and shoots stay stunted.

All tested hormone combinations led to good shoot induction response during the early stage of shoot induction from meristem. But the difference comes when the shoot starts to grow further. In this case, each treatment showed different pattern and quality of growth. All these results showed that *P. edulis* requires 1.0 mg/l BAP combined with 1.0 mg/l GA_3 and 0.1 mg/l NAA in MS solid medium to induce best shoot from meristem.

6.2. Shoot multiplication

Application of BAP and NAA decreased shooting and rooting of single nodes of Agria and Marfona cultivars of potato cultured in MS medium. The best medium for rooting and shooting was modified solid MS medium without NAA and BAP (Armin *et al.,* 2011). But the case is different in our result. Both cytokinins perform well in multiple shoot induction and Kinetin exhibited the highest shoot number compared to BAP.

Report by Badoni and Chauhan (2009) shows that GA_3 + NAA is best for shoot regeneration in multiplication of potato cultivar Kufri Himalini in comparison to the Kinetin + NAA on MS medium. In other study on medicinal plant *Ocimum gratissiumum* by Gopi *et al.* (2006), the same result was reported. Among cytokinins, 0.5 mg/l BAP responded well compared to Kinetin in medium for shoot proliferation and effect of kinetin was very less compared to BAP in shoot elongation, whereas result of this study revealed that the multiple shoot formation was better in KIN compared to BAP (alone or in combination with NAA). This was in agreement with the

results of Salehi (2006) who reported the best shoot proliferation medium were MS containing 3.0 mg/l kinetin and 0.5 mg/l NAA or 1.0 mg/l BA and 1.0 mg/l NAA for cultivars of *Dianthus caryophyllus* species. On the other hand, using BAP (0.5 mg/l) alone was found to be enough for high number of shoot formation (Nagib *et al.,* 2003). Higher concentration of BAP (2.0 mg/l) was reported to be best for induction of maximum number of shoots in potato (14 shoots per explant) by Rabbani *et al.* (2001).

On the other hand, Badoni and Chauhan (2009) reported that combination of higher concentration of Kinetin (1.0 mg/l) and low concentration of NAA responded the least mean shoot height and number of nodes in potato cultivar Kufri Himalini. In the present study, the highest mean number of multiple shoots were observed in 1.0 mg/l KIN in combination with 0.1 mg/l NAA for Holeta and 3.0 mg/l KIN in combination with 0.05 mg/l NAA for Welayta, whereas this was in agreement with the results of Hoque (2010) who reported that the maximum shoot induction percentage and highest number of shoots on other cultivars of potato was observed in medium supplemented with 2.0 mg/l IAA in combination with 2.0 mg/l KIN. Tekalign Wondimu *et al.* (2012) obtained four shoots per nodal explants from meristem derived single nodes cultured on MS medium containing 0.5 mg/l BAP in combination with KIN for four different cultivars of sweet potato.

P. edulis treated with PGR free medium exhibited strong apical dominance with little tendency to branch. Due to this, most of the control had only single shoot and mean length of shoot was maximum which may be because of apical dominance. This is in agreement with findings of Ezeibekwe *et al.* (2009) who reported in white Yam that shoots cultured on PGRs free medium gave the longest shoot.

The result of this study revealed that the number of shoots increased and the mean length of shoots decreased as the concentration of BAP increased. Similar result on potato cultivar was found by Kazemiani *et al.* (2012) as they reported the number of lateral shoots was increased by using 2.0 mg/l BAP, but main shoot length was declined by addition of BAP on culture media. Medium with the highest cytokinin concentration showed the maximum number of multiple shoots and lowest length of shoots. This may be due to the fact that suppression of apical dominance leads to the production of more number of multiple shoots and reduced shoot length.

A comparatively lower response was recorded when BAP or Kinetin was added alone in the medium in Water Yam (Behera *et al.,* 2009). In our study, when both types of cytokinin were used alone, they produced multiple shoots and KIN was slightly more effective. When BAP or Kinetin combined with NAA in multiplication medium, the frequency of multiple shoot development was significantly improved as indicated by significant difference in the number of shoots between explants treated with 1.0 mg/l BAP alone and its combination with 0.05 mg/l NAA (Table 3). This was in agreement with the report of Adeniyi *et al.* (2008) as there was significant BAP x NAA interaction indicating that the effectiveness of each of the phytohormones in inducing shoots was influenced by the presence or absence of the other. The work of Hoque (2010) also supports this finding as cytokinin group of hormone (KIN) can regenerate shoot but the rate of regeneration was little higher in combination with auxin (IAA).

Therefore, inducing multiple shoot production using Kinetin could increase multiplication rate and cost effectiveness of micropropagation to mass produce planting materials.

6.3. Rooting and acclimatization

In this study, among the *in vitro* rooting cultures the maximum number of roots was observed in MS medium combined with 1.0 mg/l IBA and even better in half strength MS medium. There was statistically significant difference in percentage of shoots rooted observed in full and half strength MS medium of the same IBA concentration ($P \geq 0.05$). Rooting efficiency was improved when salt concentration was reduced by half. Ahmad *et al.* (2003) reported as the best root system was developed on half strength MS medium supplemented with 3.0 mg/l IBA. Espinosa *et al.* (2006) also reported the highest rooting percentage of *Prunus serotina* (27%) from adventitious shoots and number of roots per shoot (2.3 ± 0.2) was obtained on MS medium containing 2.5 µM IBA when shoots were maintained for 7 days in the dark on rooting medium before transfer to a 16-hour photoperiod.

Our result was also in agreement with the results of Nagib *et al.* (2003) who recommended single use of IBA (0.5 mg/l) for proper root and shoot developments from primary meristem of potato cultivar. The auxins, NAA and IBA were used by Behera *et al.* (2009) to induce rooting from *in vitro* raised shoots of white yam. In their study half strength MS basal medium containing NAA (2.0 mg/l) has best rooting response than IBA (2.0 mg/l).

However, the overall best result in rooting was observed in *ex vitro* rooting condition. Even if, *ex vitro* rooting was shown lower percent of survival (89.6 %, 76.9 % from Holeta and Welayta respectively) compared to acclimatization of rooted shoots (100 % and 96 % from Holeta and Welayta respectively), it showed the highest result in percentage root induction, number of roots

per explant and length of root. It was promising considering the reduction in cost by avoiding the *in vitro* rooting and the reduction in labor and time of establishment from laboratory to field.

Ex vitro rooting has also been reported in *Mentha piperita* L. (Sunandakumari *et al.,* 2004). The optimum growth rate of deflasked plantlets frequently does not occur until new leaves and roots develop in the green-house environment (Seelye *et al.,* 2003). The roots formed in agar have very few root hairs and behave like "water roots" (Hollings, 1965). In these cases, early works in developing rooted shoots *in vitro* have minimal importance. Reducing the cost of *in vitro* production is a key issue for increasing the application of the method. Rooting of microcuttings *in vitro* is expensive and can even double the price of the cutting. Another difficulty is to prepare rooted plants for planting them *ex vitro*. Sometimes it is hard to remove agar from roots without excessive damage that invite facultative microbial pathogens (Hollings, 1965).

Considering reduction in time and resource *ex vitro* rooting was more favourable than *in vitro* rooting. Moreover, study in *Mentha piperita* L. shows that plantlets established in soil were similar in morphological characters to that of mother plants (Sunandakumari *et al.,* 2004). The result of this study also shows the fact that *ex vitro* rooting of *P. edulis* can efficiently replace *in vitro* rooting works. Therefore, using *ex vitro* rooting makes the protocol very economical.

Analyzing the results of the present experiment, it is possible to state that in case of *P. edulis*, full strength and half strength MS medium combined with 1.0 mg/l IBA and *ex vitro* rooting by dipping shoots in 5.0 mg/l IBA for 5 minutes led to very good rooting response, particularly remarked was the use of *ex vitro* rooting as it was resulted in best rooting response with minimal cost.

There was statistically significant difference between the locals from two growing area in rooting performance. Explants from Holeta local showed better rooting percentage and number of roots than those of Welayta local.

Acclimatization of *in vitro* rooted plantlets was highly successful, in that most plants survived. All explants of Holeta local and 96% of Welayta local were survival and established as healthy plant. Similar results were observed by Tekalign Wondimu *et al.* (2011) in sweet potato as 100% survival was observed.

7. Conclusions

With the utilization of the proper rapid multiplication techniques, manipulating *in vitro* condition and growth regulator applications, the availability of high quality plantlets of *P. edulis*, which serves as planting material, can be enhanced. Based on result of this study, the following conclusions were reached:

- Results achieved in this study demonstrate that MS medium containing 0.1 mg/l NAA, 1.0 mg/l GA_3 and 0.1 and 1.0 mg/l BAP induced maximum percent of shoots from meristem.
- Nodal shoot explants gave maximum shoot number on MS medium containing 1.0 mg/l KIN in combination with 0.1 mg/l NAA and 3.0 mg/l in combination with 0.05 mg/l from Holeta local and Welayta local, respectively.
- Percentage of rooting, number of roots per explant and root length were best on *ex vitro* rooting condition; planting micropropagated shoots by first dipping them in to 5.0 mg/l IBA for 5 minutes.
- Acclimatization was highly successful for all plantlets of Holeta and 96% of Welayta local survived in the soil mix and established as healthy plants.

8. Recommendations

Based on the results of this study the following recommendations were forwarded:

- Micropropagation cost could possibly be reduced further with the substitution of the other high cost components of the culture medium. Therefore, it is important to test direct rooting of cuttings without the use plant growth hormones.
- The currents study focus on micropropagation *P. edulis*, and therefore, it is recommended to study *in vitro* tuberization efficiency of the plant so that it can serve as alternative source in solving shortage of seed tuber.
- As the components of medium for shoot initiation, multiplication and rooting are expensive, low cost micropropagation methods such as using table sugar instead of sucrose should be used for this plant in the future.
- There are local cultivars of *P. edulis* identified by farmers but they were not well documented and found in research center. Therefore, it is important to collect and document existing local cultivars all over the country so that propagation protocol based on each cultivar will be developed.

9. References

Acedo, V. A. (2006). Improvement of *in vitro* techniques for rapid meristem development and mass propagation of Philippine cassava (*Manihot esculenta* Crantz). *J. food Agric. Environ*. **4**(1): 220-224.

Adeniyi, O. J., Adetimirin, V. O., Ingelbrecht, I. and Asiedu, R. (2008). Shoot and plantlets regeneration from meristems of *Dioscorea rotundata* Poir and *Dioscorea alata* L. *Afr. J. Biotechnol.* **7**(8): 1003-1008.

Adesoye, A. I., Okooboh, G. O., Akande, S. R., Balogun, M. O. and Odu, B. O. (2012). Effect of phytochromones and genotype on meristem and shoot tip culture of *Telfairia occidentalis* Hook F. *J. Appl. Biosci*. **49**: 3415-3424.

Ahmad, T., Ur-Rahman, H., Ahmad, C.M. and Laghari, M. H. (2003). Effect of culture media and growth regulators on micropropagation of peach rootstock GF 677. *Pak. J. Bot*. **35**(3): 331-338.

Alam, I., Akhtar, S. S., Kamrun, N. M., Jahangir, A. M., Anisuzzaman, M. and Firoz, A. M. (2010). Effect of growth regulators on meristem culture and plantlet establishment in sweet potato (*Ipomoea batatas* (L.) Lam.). *Plant Omic. J*. **3**(2): 35-39.

Altaf, N. (2009). Comparative study of meristem and callus regenerated lentil (*Lens culinaris* Medik) plants. *Electronic J. Environ. Agric. Food Chem*. **8**(1): 76-80.

Armin, M. J., Asgharipour, M. R. and Yazdi, S. K. (2011). Effects of different plant growth regulators and potting mixes on micropropagation and mini-tuberization of potato plantlets. *Adv. Environ. Biol*. **5**(4): 631-638.

Badoni A. and Chauhan J. S. (2009). Effect of growth regulators on meristem-tip development and *in vitro* multiplication of potato cultivar 'Kufri Himalini'. *Nat. Sci*. **7**(9): 31-34.

Behera, K. K., Sahoo, S. and Prusti, A. (2009). Rapid in vitro micropropagation of sugarcane (*Saccharum officinarum* L. cv-*Nayana*) through callus culture. *Nat. Sci*. **7**(4): 1-10.

Bhojwani, S. S. and Razdan, M. K. (1996). Plant Tissue Culture: Theory and Practice, A Reviesed Edition. Elsevier, Amsterdam.

Brown, D. W. and Thorpe, T. A. (1995). Crop improvement through tissue culture. *World J. Microbiol. Biotechnol*. **11**: 409-415.

Bryan, A. D., Vansbroeck, Z. P., Schultheis, J. R., Pecota, K. V., Swallow, W. H. and Yencho, G. C. (2003). Cultivar decline in sweet potato: I. Impact of micropropagation on yield, storage root quality and virus incidence in Beauregard. *J. Amer. Soc. Hort. Sci*. **128**(6): 846-855.

Bryan, J. E. (1983). The importance of planting material in root and tuber crop production. **In**: *Global Workshop on Root and Crop Propagation, Proceedings of Regional Workshop held in Cali, Colombia, 13-16 September.* pp. 4. (James H. C., ed). Colombia.

Bula Sirika (2011). A quest for traditional uses of coffee among Oromo people with special emphasis on Wallaga, Ethiopia. *Afr. Diaspora Archaeol. Network September 2011 Newsletter*.

Dagnino, D. S., Carelli, M. D., Arrabal, R. F. and Esquibel, M. A (1991). Effect of Gibberellic acid on *Ipomoea batatas* regeneration from meristem culture. *Pesq. Agropec. Bras. Brasflia*. **26**(2): 259-262.

Dandena Gelmesa (2010). Shifting to alternative food source: potential to overcome Ethiopias' malnutrition and poverty problems. Agriculture and Food. Montpellier, France.

Dangler, J. M. (1994). Sweet potato foundation programs provide sweet potato growers with high quality pathogen-free seed stock. *Hort. Technol*. **4**(3): 224-227.

Dawit Beyene (2009). Micropropagation of Selected Cassava Varieties (*Manihot esculenta* Crantz) from Meristem Culture. M. Sc. Thesis. Addis Ababa University, Addis Ababa.

Dodds, J. H. and Roberts, L. W. (1985). Experiments in Plant Tissue Culture. Cambridge University Press, New York.

Espinosa, A. C., Pijut, P. M. and Michler, C. H. (2006). Adventitious shoot regeneration and rooting of *Prunus serotina in vitro* cultures. *Hort. Sci*. **41**(1): 193-201.

Ezeibekwe, I.O., Ezenwaka, C.L., Mbagwu, F.N. and Unamba, C.I.N. (2009). Effect of combination of different levels of Auxin (NAA) and Cytokinin (BAP) on *in vitro* propagation of *Dioscorea rotundata L.* (white Yam). *New York Sci. J*. **2**(5): 1554- 1564.

George, E. F. and Debergh, P. D. (2008). Micropropagation: Uses and methods. **In**: *Plant Propagation by Tissue Culture*. *Volume 1. The Background*. pp. 29-64. (Edwin F. G., Michael A. H. and Geert-Jan D. K., eds) Springer, Dordrecht.

Gopi, C., Sekhar, Y. N. and Ponmurugan, P. (2006). *In vitro* multiplication of *Ocimum gratissiumum* L. through direct regeneration. *Afr. J. Biotechnol*. **5**(9): 723-726.

Haapala, T. (2004). Establishment and use of juvenility for plant propagation in sterile and non-sterile conditions. Ph. D. Thesis. University of Helsinki, Helsinki.

Hanna Beksissa (2006). A study on the response of three genotypes of *Mucuna pruriens* to different growth media for tissue culture differentiation. M. Sc. Thesis. Addis Ababa University, Addis Ababa.

Harisha, S. (2007). Biotechnology Procedures and Experiments Handbook. Infinity Science Press LLC, Hingham.

Hedberg, I., Ensermu Kelbessa, Edwards, S., Sebsebe Demissew and Persson, E. (2006). *Flora of Ethiopia and Eritrea*, Vol. 5. The National Herbarium. Addis Ababa, Ethiopia.

Hollings, M. (1965). Disease control through virus-free stock. *Annu. Rev. Phytopathol*. **3**: 367-396.

Honda, H., Lui, C. and kobayashi, T. (2001). Large-scale plant micropropagation. **In**: *Advances in Biochemical Engineering Biotechnology, Plant Cells* (Zhong, J. J. ed). Springer, Shanghai.

Hoque M. E. (2010). *In vitro* regeneration potentiality of potato under different hormonal combination. *World J. Agric. Sci*. **6**(6): 660-663.

IBC (Institute of Biodiversity Conservation). (2007). Country Report on the State of Plant Genetic Resources for Food and Agriculture. Addis Ababa.

IBC (Institute of Biodiversity Conservation). (2007). Ethiopia: Second Country Report on the State of Plant Genetic Resources for Food and Agriculture to FAO. Addis Ababa.

IBC (Institute of Biodiversity Conservation). (2005). Ethiopia Third National Report. Addis Ababa.

IBC (Institute of Biodiversity Conservation). (2005). National Biodiversity Strategy and Action Plan. Addis Ababa.

IITA (International Institute of Tropical Agriculture). (2008). Medium Term Plan 2009-2011. International Potato Center. Ibadan, Nigeria.

Ivan, I., Alena, G., Gabriela L. and Shri M. Jain (2010). Plant micropropagation. **In:** *Plant Cell Culture* (Michael R., Davey and Paul A., eds). John Wiley and Sons, Ltd.

Jha, T. B. and Biswajit G. (2005). Plant Tissue Culture: Applied and Basic. Universities Press, India.

Kalilian, F. H. and Faramarz, F. (1990). Hormonal control of growth and development. **In**: *Methods in Molecular Biology, Volume 6, Plant Cell and Tissue Culture*, (Walker, J. M., ed). Humana Press, New Jersey

Kartha, K. K. and Gamborg, O. L. (1975). Elimination of Cassava Mosaic Disease by Meristem Culture. *Phytopathol*ogy **65**: 826-828.

Kazemiani, S., Motallebi-Azar, A., Mohaddes, N., Kiomarsy, F., Yarmohammadi, F. and Etedali, F. (2012). Effect of different concentrations of sucrose and BAP on shoot proliferation on MS strength basal media in potato Cv. Agria. *Southwest. J. Hort. Biol. Environ*. **3**(1): 63-72.

Koehorst-van Putten, H. J., Sudarmonowati, E., Herman, M., Pereira-Bertram, I. J., Wolters, A. M., Meima, H., de Vetten, N., Raemarkers, C. J. and Visser, R. F. (2012). Field

testing and exploitation of genetically modified cassava with low-amylose or amylase-free starch in Indonesia. *Transgen. Res*. **21**: 39-50.

Loyola-Vargas, V. and Vazquez-Flota, F. (2006). An introduction to plant cell culture: Back to the future. **In**: *Methods in Molecular Biology, Plant Cell Culture Protocols* (Loyola-Vargas, V. and Vazquez-Flota, F. eds). Humana Press, New Jersey.

MAFF Research Council (1994). The first ministry of Agriculture, forestry and fisheries, Japan international workshop on genetic resource: Root and tuber crops. Japan International Research Center for Agricultural Science, Japan.

Moa Megersa (2010). Ethnobotanical Study of Medicinal Plants in Wayu Tuka Wereda, East Wollega Zone of Oromia Region, Ethiopia. M. Sc. Theses. Addis Ababa University, Addis Ababa.

Mulugeta Taye, Lommen, W. J. M. and Struik, P. C. (2007). Indigenous multiplication and production practices for the tuber crop *Plectranthus edulis* in Chencha and Welaita, Southern Ethiopia. *Expl. Agric*. **43**:381–400.

Mulugeta Taye (2008). Studies on agronomy and crop physiology of *Plectranthus edulis* (Vatke) Agnew. Ph. D. Thesis. Wageningen University, Wageningen.

Murashige, T. (1974). Plant propagation through tissue cultures. *Annu. Rev. Physiol*. **25**: 135-166.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco cell cultures. *Physiol. Plant*. **15**: 473-497.

Mutandwa, E. (2008). Performance of tissue cultured sweet potatoes among smallholder farmers in Zimbabwe. *Agric. Biol. For.* **11**(1): 48-57.

Nagib, A., Hossain, S. A., Alam, M. F., Hossain, M. M., Islam, R. and Sultana, R. S. (2003). Virus free potato tuber seed production through meristem culture in Tropical Asia. *Asian J. Plant Sci*. **2**(8): 616-622.

Okereke, C. O. (2012). Growth performance of Finisher broilers on Livingstone potato (*Plectranthus esculentus*) tubers meal. *Pak. J. Nutr*. **11**(5): 411-413.

Otroshy M. (2006). Utilization of tissue culture techniques in a seed potato tuber production scheme. Ph. D. Thesis. Wageningen University, Netherlands.

Omar, M. S. and Aouine, M. (2007). Commercial *in vitro* mass propagation of plant: Current status and future investment prospects. *Sirt. Turk. J. Biol*. **2**(4): 94-99.

Padulosi, S. T., Hodgkin, J. T., Williams and Haq, N. (2002). Underutilized crops: trends, challenges and opportunities in the 21st Century. **In**:"*Managing plant genetic resources*" pp.15 (Engels J.M.M. *et al.,* eds.) CABI-IPGRI. Southampton, UK.

PGRC (Plant Genetic Resource Center). (1996). Ethiopia: Country Report to the FAO International Technical Conference on Plant Genetic Resources. Addis Ababa.

Puonti-Kaerlas, j. (1998). Cassava Biotechnology. *Biotechnol. Genet. Eng. Rev*. **15**: 329-364.

Rabbani, A., Askari, B., Abbasi, N. A., Bhatti, M. and Quraishi, A. (2001). Effect of growth regulators on *in vitro* multiplication of potato. *Int. J. Agric. Biol*. **3**(2): 181-182.

Ryan, J. (2002). Understanding and Managing Cell Culture Contamination, Technical Bulletin. Corning Incorporated Press, France.

Salehi, H. (2006). Can a general shoot proliferation and rooting medium be used for a number of carnation cultivars. *Afr. J. Biotechnol*. **5**(1): 25-30.

Seelye, J. F., Burge, G. K. and Morgan, E. R. (2003). Acclimatizing tissue culture plants: Reducing the shock. *Com. Proc. Int. plant prop. Soc.* **53**: 85-90.

Sreedhar, R. V., Venkatachalam, L. and Bhagyalakshmi, N. (2007). Genetic fidelity of long term micropropagated shoot cultures of vanilla (*Vanilla planifolia,* Andrews) as assessed by molecular markers. *Biotechnol. J*. **2**: 1007-1013.

Stepan, S. G. (1990). Selection of media for tissue and cell culture. **In**: *Methods in Molecular Biology, Volume 6, Plant Cell and Tissue Culture*, (Walker, J.M., ed). Humana press, New Jersey.

Sunilkumar, G.S. (2005). Influence of organic manures and growth regulators on growth and yield of *Coleus* (*Coleus forskohlii Briq*.). M. Sc. Thesis. University of Agricultural Sciences, Dharwad.

Sunandakumari, C., Martin, K. P., Chithra, M., Sini, S. and Madhusoodanan, P. V. (2004). Rapid axillary bud proliferation and *ex vitro* rooting of herbal spice, *Mentha piperita* L. Indian *J. Biotechnol.* **3**: 108-112.

Tekalign Wondimu, Tileye Feyissa and Girma Bedada (2012). Meristem culture of selected sweet potato (*Ipomoea batatas* L. Lam) cultivars to produce virus-free planting material. *J. Horti. Sci. Biotechnol*. **87**(3): 255-260.

Weyessa Garedew, Admasu Tsegaye, Bezuayehu Tesfaye and Hussein Mohammed (2009). Variability and association of quantitative traits in *Plectranthus edulis* (Vatke) Agnew. *East Afr. J. Sci*. **3**(1): 61-69.

Yasmin, A., Jalbani, A. A. and Raza, S. (2011). Effect of growth regulators on meristem tip culture of local potato CVs desiree and patrones. *Pak. J. Agril. Engg. Vet. Sci*. **27**(2): 143-149.

Yeshitila Mekbib (2007). Phenotypic variation and local customary use of Ethiopian potato (*Plectranthus edulis* (Vatke) Agnew). M. Sc. Theses. Uppsala University, Sweden.

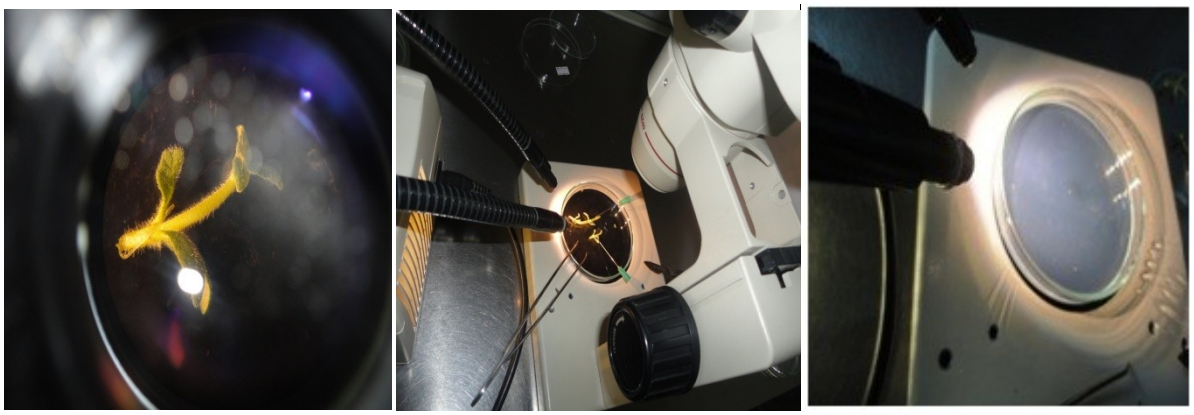
10. Appendices

Appendix 1. Stock solution for MS (Murashige and Skoog's, 1962)[a].

Macronutrients	g/l	Micronutrients	mg/500ml	Vitamins	mg/500ml
KNO_3	38	$FeSO_4.7H_20$	2780	Myoinositol	10000
NH_4NO_3	33	$Na_2EDTA.2H_20$	3730	Glycine	200
$CaCl_2.2H_2O$	8.8			Nicotinic acid (NaOH)	50
$MgSO_4$	7.4	$ZnSO_4.7H_2O$	430	Pyridoxine (B6)	50
KH_2PO_4	3.4	H_3BO_3	630	Thiamin (B1)	10
		$MnSO_4.\ 4H_2O$	2230		
		$CuSO_4.\ 5H_2O$	2.5		
		KI	83		
		$Na_2MoO_4.\ 2H_2O$	25		
		$CoCl_2.\ 6H_2O$	2.5		

[a]To prepare 1L of medium, take 50ml of Macro, 5 ml of Iron-EDTA, 5 ml of Micro and 5ml of Vitamin.

Appendix 2. Isolation and transfer of meristematic tissue of *P. edulis* in to culture medium.



Appendix 3. Data on number of explants gave multiple shoots and simultaneous rooting

Treat code	No. of explants gave multiple shoot				No. of explants showing simultaneous rooting			
	Holeta		Welayta		Holeta		Welayta	
	No.	%	No.	%	No.	%	No.	%
Control	10	16.6	0	0	45	75	56	93.3
1	44	73.3	45	76.7	0	0	0	0
2	60	100	58	96.7	0	0	0	0
3	60	100	60	100	0	0	0	0
4	47	78.3	52	85	10	16.7	4	6.7
5	19	68.3	56	95	0	0	0	0
6	60	100	60	100	0	0	0	0
7	44	75	52	86.7	0	0	10	16.7
8	60	100	60	100	0	0	0	0
9	60	100	60	100	9	15	6	10
10	52	86.7	52	85	8	13.3	6	10
11	54	90	60	100	4	6.7	0	0
12	60	100	60	100	9	15	12	20
13	60	100	60	100	0	0	0	0
14	52	86.7	56	93.3	0	0	0	0
15	57	95	54	90	0	0	0	0
16	60	100	60	100	0	0	0	0
17	60	100	60	100	0	0	0	0
18	60	100	58	98.3	0	0	0	0
19	60	100	56	93.3	18	30	12	20
20	60	100	58	96.7	0	0	0	0
21	60	100	60	100	0	0	0	0

60 explants per treatment were used

Appendix 4. Data showing number of explants rooted

Treatments code	Explant rooted			
	Holeta local		Welayta local	
	Number	%	Number	%
Control	36	60	19	31.7
1	18	30	21	35.0
2	6	10	20	33.3
3	57	95	37	61.7
4	15	25	6	10
5	60	100	46	76.7
6	23	38.3	10	16.7
Ex vitro	60	100	60	100

60 explants per treatment were used

Appendix 5. Number of shoots (direct cuttings) survived in glasshouse

	Shoots survived	
	Number	%
Holeta local	54	90
Welayta local	46	76.6

60 explants per treatment were used

Appendix 6. Number of *in vitro* rooted shoots survived in glasshouse

	Shoots survived	
	Number	**%**
Holeta local	60	100
Welayta local	58	96.6

60 explants per treatment were used

Appendix 7. ANOVA table on the effect of two localities in shoot induction from meristem

ANOVA

MrShoot					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.750	1	6.750	.042	.841
Within Groups	1591.500	10	159.150		
Total	1598.250	11			

Appendix 8. ANOVA table on the effect of two localities in shoot multiplication and simultaneous rooting

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ShootNo.	Between Groups	11.843	1	11.843	2.184	.140
	Within Groups	14168.922	2613	5.422		
	Total	14180.765	2614			
PerMShot	Between Groups	673.578	1	673.578	1.692	.193
	Within Groups	1040743.325	2614	398.142		
	Total	1041416.903	2615			
PerRt	Between Groups	11.160	1	11.160	.034	.854
	Within Groups	864724.119	2614	330.805		
	Total	864735.278	2615			

Appendix 9. ANOVA table on the effect of two localities in rooting

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
NoRt	Between Groups	73.775	1	73.775	15.833	.000
	Within Groups	4524.295	971	4.659		
	Total	4598.070	972			
PerRt	Between Groups	31580.342	1	31580.342	31.157	.000
	Within Groups	984182.445	971	1013.576		
	Total	1015762.787	972			