

Adama Science and Technology University

School of Natural and Applied Natural Sciences



**Protocol Development for Micropropagation of an Elite Black
Pepper (*Piper nigrum* L.) Cultivar: SI -3/80**

By

Tilahun Hailu-

Seifu Juneidi

May, 2017

Adama, Ethiopia

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LIST OF ABBREVIATIONS

| | |
|--------|--|
| AC | Activated Charcoal |
| APG | Angiosperm Phylogenic Group |
| BA | N6-Benzyl adenine |
| BAP | 6-Benzylaminopurine |
| CRD | Completely Randomized Design |
| 2, 4-D | 2, 4- Dichlorophenoxyacetic acid |
| EIAR | Ethiopian Institute of Agricultural Research |
| HCl | Hydrochloric acid |
| IAA | Indoleacetic acid |
| IAR | Institute of Agricultural Research |
| IBA | Indole 3-butyric acid |
| JARC | Jimma Agricultural Research Center |
| KN | Kinetin |
| MS | Murashighe and Skoog |
| NAA | Naphthaleneacetic acid |
| PGRs | Plant Growth Regulators |
| REGWO | Ryan, Elinot, Gabriel, and Welsh model |
| SAS | Statistical Analysis Software |
| TNSRC | Teppi National Spices Research Center |

ABSTRACT

Black pepper (Piper nigrum L.) is a perennial woody climbing shrub that belongs to the family Piperaceae. It is one of the oldest and most important spices, and is still used universally in food flavoring. It is christened as the 'king of spices'. The spiciness and quality of black pepper is contributed by two chemical components: piperine ($C_{17}H_{19}NO_3$) contributes to pungency while pepper oleoresin and essential oil is responsible for the aroma and flavor. Accession SI-3/80, which was introduced from abroad and adapted well in Ethiopia, is an improved hybrid cultivar with a relatively high yield and good quality. Lack of an adequate supply of high quality planting material is the major bottle neck problem in conventional propagation method. Plant tissue culture is a biotechnological approach to alleviate this problem. Therefore, this study was initiated with the objective of developing an efficient and reproducible in vitro mass propagation protocol for 3/80 cultivar of P. nigrum L. through micropropagation using shoot tip explants. Three experiments were carried out in this study. All the experiments were laid in CRD with factorial treatment combinations of four replications, five explants per jar under each replicate. The experiments were laid after sterilization of shoot tip explants with 15% berekina for 20 minutes. For shoot induction experiment, maximum percentage $62.50 \pm 0.00\%$ was observed on half MS medium supplemented with 2mg/l BAP. In shoot multiplication experiment, $\frac{1}{2}$ MS + 2mg/l BAP + 1.5mg/l KN resulted in 4.60 ± 0.00 shoot number with best vigor and morphological appearance. In the in vitro rooting experiment best shoot elongation and rooting condition was recorded on $\frac{1}{2}$ MS + 1 mg/l IBA which resulted mean values of 8.20 ± 0.00 root number, 6.33 ± 0.10 cm root length and 4.45 ± 0.75 cm shoot height followed by $\frac{1}{2}$ MS + 1 mg/l NAA with 6.41 ± 0.05 root number, 6.10 ± 0.00 cm root length and 3.52 ± 0.75 cm shoot length. The results obtained in this study permit the development of mass propagation protocol using shoot tip explant sources that could enable large scale commercial production of this highly demanded 3/80 cultivar of P. nigrum L. true-to type and provide a possible system towards genetic improvement of the crop.

Key-words/phrases: Artemisinin, Explant, Microcutting, Microshoot, Plant growth regulators, Plantlet

1. INTRODUCTION

1.1 Background

Black pepper (*Piper nigrum* L.) is a perennial woody climbing shrub native to the south-western coastal regions of India and is now grown in many countries, particularly in tropics. It is a member of the Piperaceae family with a somatic chromosome number of $2n=52$ (Gilbert, 2000, Krishnamoorthy and Parthasarathy, 2010). It is one of the oldest and most important spices used universally in food flavoring. The whole fruit (drupe) is extensively used as a condiment and flavoring for all kinds of savory dishes, in preserving and pickling, in the manufacture of sauces, medicines and perfumery (Nazeem *et al.*, 2004). The crop is generally christened as the 'king of spices' (Mathew *et al.*, 2001). The spiciness and quality of black pepper is contributed by two chemical components: piperine ($C_{17}H_{19}NO_3$) contributes to pungency while pepper oleoresin and essential oil is responsible for the aroma and flavor (Ravindran, 2000).

Black pepper is grown in about 26 countries of the world, with a production of 369,587 ton from 467,708 hectare having a productivity of 790.2 kg per hectare. The area of production and productivity vary among countries. However, a steady increase in area and production was noted from year to year (Krishnamoorthy and Parthasarathy, 2010). Vietnam's yearly production is about 100,000 ton followed by India producing 60,000 to 70,000 ton per year. USA, Denmark, Germany, Belgium and Netherlands are the leading importers (Sreekumar, 2008). Madagascar, Malawi, Zimbabwe, Benin and Kenya are black pepper producing countries of Africa but not recognized in international export market rather than for local consumption and domestic market (Nazeem *et al.*, 2004). In terms of monetary value, peppercorns (dried black pepper) are the most widely traded spice in the world, accounting for more than 30 % of the total spices traded internationally. For instance, India's average export earning is reported to be about 32.7 million dollar per year (Singh and Singh, 2007).

The crop requires a hot, humid tropical climate with a long rainy season and it is more productive when grown under shades and provided with some sort of support (Cobley, 1979). Over 100 black pepper cultivars are known worldwide. Among which accession SI-3/80, which was introduced from abroad and adapted well in Ethiopia, is an improved hybrid cultivar with a

relatively high yield and good quality. This cultivar is more popular throughout the pepper growing tracts of the world (Krishnamoorthy and Parthasarathy, 2010).

Black pepper, which is known by the name 'yebahir kimem' or 'kundo-berbere' in Ethiopia, is used in all parts of the country. The dried fruits are thought to be imported to Ethiopia and are expensive in local markets (Edossa, 1998). The plant material was introduced to the country between the years 1979 to 1980 and was established at Teppi, Jimma and Bebek sites for evaluation. Of the total introduced accessions, fourteen had revealed better adaptation and promising performance under the Teppi and Bebek conditions. Sl 3/80 or "Tato" and Pa 4/80 or "Gacheb" are cultivars released nationally for their better yield and quality as an outcome of selection work done on those adapted accessions of *P. nigrum* (Edossa, 1998; Girma *et al.*, 2008). The average yield of Sl-3/80 cultivar as recorded from Bebek and Teppi, is appreciable as compared to the international average level. Three years average yield is reported as about 2.8 kg fresh yields per vine and 2 ton dry yield per hectare. The chemical analysis of oleoresin yield, volatile oil and piperine content of Bebek's black pepper, as reported by Berhanu and Nigist (1998), indicated that the Ethiopian black pepper satisfied international standard quality of 4-7% of piperine content. Therefore, it had been concluded that localities having similar agro-ecologies with that of Teppi and Bebek, i.e. hot and humid areas with well distributed high annual rainfall and low land altitudes at or below 1250m.a.s.l, to be suitable for black pepper cultivation (Engles *et al.*, 1991; Ethiopian Investment Agency, 2010).

Despite the prevalence of wide areas with suitable agro ecologies and availability of best varieties, Ethiopia had not benefited from its cultivation so far. Except the two state coffee farms of Teppi and Bebek, the crop had not been disseminated to the smallholder farmers that contribute the majority of national agricultural production. This is ascribed to varied problems, primarily due to shortage of planting materials. In this regard, Philip *et al.*, (1992) contrasted that, the use of conventional cuttings with 2-6 nodes for nursery and field plantations can yield only 50 cuttings per plant per year, limiting the potential to proliferate improved varieties as compared to the recorded *in vitro* proliferation rate that would allow the production of about 15,000 plants from a single shoot tip per year. In addition, the necessity for obtaining stem cuttings from different vines brings about variation in growth habit and productivity.

Major constraints in the cultivation of black pepper through the conventional propagation method are its low productivity due to poor genetic stock, incidence of pests and diseases and its latency (Mathew *et al.*, 2001). Growing *P. nigrum* is difficult to start from seed and is easier to start from a stem cutting (Mathew *et al.*, 2001). Reproduction via seed is uncertain and yields only few heterogeneous progenies due to their short viability and high sterility in post generation stages (Abassi *et al.*, 2010). As germination and the raising of young plants from seeds takes too long, seed propagation of black pepper is out of question for commercial plantations. The seeds are also quite possibly dormant with their viability severely affected due to storage (Chaundhury and Chandel, 1994). According to Ravindran (2000) and Nazeem *et al.* (2004), the conventional stem cutting and seed propagation method of black pepper is highly affected by foot rot disease that is caused by *Phytophthora capsici* and could result in severe vine mortality and crop losses of 20 to 30%, affecting almost all of the cultivars available.

In vitro propagation is an alternative method to the conventional horticultural propagation using cuttings. *In vitro* culture offers improvements over the traditional vegetative propagation, as it enables rapid rate of clonal multiplication of an elite plant species, allowing production of disease-free, genetically stable and uniform progenies (Hu and Wang, 1983). A number of works have been reported on *in vitro* multiplication of *P. nigrum* varieties using different explants (Philip *et al.*, 1992; Babu *et al.*, 1993; Nair and Gupta, 2006; Hussain *et al.*, 2011). Philip *et al.* (1992) showed that, multiple shoots can be induced from shoot tip explants of mature vine using BAP alone (0.5-3 μM) or in combination with auxins in the culture media and *in vitro* developed shoots could be easily rooted using auxins (0.5-1.5 μM) in 50% strength MS basal media.

To bring production of pepper to a commercial level in Ethiopia, conventional vegetative propagation would be ineffective and time consuming. This is mainly attributed to the limited number of stock plants available that make collection of stem-cuttings nearly impossible. Therefore, to increase the area of cultivation and maximize the economic benefits for the growers and to enhance the country's foreign currency earnings from its export, large scale propagation that meets the prevailing huge demand for propagules is highly essential. This calls for the use of the modern tissue culture technique that enables production of large number of plantlets from a single mother plant within a relatively shorter period of time (FAO, 2009).

With this regard, as there is no protocol that has been optimized for *in vitro* propagation of this nationally released black pepper cultivar SI-3/80 in Ethiopia, this proposal is initiated to address this critical issue, thereby boost its production through enhanced dissemination of clean planting materials.

1.2. Objectives

1.2.1. General objective:

- ❖ To develop an efficient protocol that enables rapid *in vitro* multiplication of an elite Ethiopian black pepper (*P. nigrum* L) cultivar SI-3/80.

1.2.2. Specific objectives:

- ✚ To find out the optimum concentration of cytokinins and strengths of MS basal media for effective culture initiation.
- ✚ To establish the optimum concentration of cytokinins for effective *in vitro* shoot multiplication.
- ✚ To determine the optimum combinations and concentrations of auxins for effective shoot and root growth and development.

2. LITERATURE REVIEW

2.1 Botanical description of the plant

Black pepper (*P. nigrum* L) is a flowering vine in the family Piperaceae, which is cultivated for its fruit. The Piperaceae, also known as the pepper family, is a large family (APG III, 2009) that contains roughly 3,610 currently accepted species categorized under five genera. The vast majority of them are found within the two main genera: *Piper* (2000 species) and *Peperomia* (1600 species). The most well-known species of this family is *P. nigrum*, which yields peppercorns that are commonly used as spices, including black pepper, although its relatives in the family include many other spices (Ravindran, 2000; Wanke *et al.*, 2007).

The *P. nigrum* plant is a rhizomatous, perennial glabrous woody climber that grows as high as 10 m (Gilbert, 2000) clinging to its supports using ivy like roots, so as it can produce flowers and fruits. Black pepper is a vigorous vine with thick and rough old stem bearing numerous flowering plagiotropic branches. Wild forms are usually dioecious but most cultivated ones are bisexual. Many cultivated type's exhibit protogyny with female phase preceding the male phase by few days or weeks (Ravindran *et al.*, 2000; Krishnamoorthy and Parthasarathy, 2009).

The fruit is a drupe, spherical, pungent and red when ripe with a single seed per fruit. The fruit, known as a peppercorn when dried, is approximately 5 mm in diameter, dark red when fully mature, and, like all drupes, contains a single seed. Peppercorns, and the powdered pepper derived from grinding them, may be described simply as pepper, or more precisely as black pepper, white pepper, or green pepper. Green peppercorns are simply the immature black peppercorns. The odor of the fruit is penetrating and aromatic; the taste is hot, biting and very pungent (Gilbert, 2000; Ravindran *et al.*, 2000).

The crop requires a hot, humid tropical climate with a long rainy season and it is more productive when grown on well aerated loam soil under shades provided with some sort of support (Cobley, 1979). Plants require high temperature and optimum light for fruiting. *P. nigrum* blooms freely through the summer months and fruits ripening the following year. Young plants can take three to four years to come into bloom but even modest-sized plants will yield hundreds of peppercorns (Ravindran, 2000). Black peppercorns are harvested when the seeds are nearly ripe and then dried at room temperature.

2.2 Economic importance

Both black pepper and white pepper are used in cuisine worldwide, at all stages of the cooking process and as a table condiment. Pepper contains about 0.6 - 2.6% essential oil. Oil of pepper is used in the flavoring of sausages, canned meats, table sauces, soups and certain beverages. It has a number of medicinal virtues, commonly used as antacid and digestive spice (Nazeem *et al.*, 2004; Thakamani *et al.*, 2008).

In early historic times, black pepper was widely cultivated in the tropics of Southeast Asia. It is indigenous to the Malabar Coast of India. Apart from India, black pepper is widely cultivated throughout Indonesia, Malaysia, Brazil, Sri Lanka, Vietnam and China (Wanke *et al.*, 2007). Its consumption is concentrated in industrialized countries besides India. Vietnam is the largest producer of pepper, which contributes about 34% of world pepper production followed by India (19%), Brazil (13%), Indonesia (9%), Malaysia (8%), China (7%), Sri Lanka (6%) and Thailand (4%). Vietnam is also the largest pepper exporter whereas the USA is the biggest importer in the world (Thakamani *et al.*, 2008).

The most widely used form of black pepper propagation is making use of stem cuttings that are collected from the terminal areas of the main shoot. Mother plants selected for collecting cuttings should characteristically be strong, healthy and highly productive. Initially, it is imperative to remove the shoot apex, as well as all leaves and lateral shoots from the third to the seventh knots of the main shoot from those selected mother plants. Subsequently, after the plant's apex has regenerated, the shoot underneath the seventh knot will be removed and planted in a seedling bed, followed by planting of three to four knot cuttings in the soil (Purseglowe, 1977; Mathew *et al.*, 2001). In the course of time, the beds must be kept moist and shaded to create favorable growing conditions to the seedlings. Then after, the shoots will root after two months and it is common to attain around 30 % rate of success. Field transplanting can thus be carried out after about six months of nursery planting time (Nair and Gupta, 2006; Girma *et al.*, 2008).

Growing *P. nigrum* is a pain to start from seed and is easier to start from a stem cutting (Mathew *et al.*, 2001). In cases of using seeds, it is essential to soak fully-ripened berries in water for two to three days so as to remove their pulp prior to drying them under shade. Afterwards, they will be planted out in moist, shaded beds filled with a mixture of well decomposed organic matter

and sand, at a distance of five to ten cm to one-another. They will begin to germinate after 30 days, and can be transplanted to their final sites after another six months, when they produce four leaves (Ravindran *et al.*, 2000).

The black pepper plants are traditionally raised from seeds or cuttings. The productivity however is slowly declining mainly due to epidemic diseases, pests and drought which had resulted increased use of biocides, thus contaminating the produce as well as the environment (Krishnamoorthy and Parthasarathy, 2009). Shortage of improved varieties (poor genetic stock) with quality traits like significant levels of oleoresins, piperine and volatile essential oil is also a constraint too (Abbasi *et al.*, 2007).

The major bottle neck of conventional propagation of black pepper is the damage caused by fungus *Phytophthora capsici*, nematodes *Rodopholus simittis* and the pest poll- beetle *Longitarsus nigripennis* (Krishnamoorthy and Parthasarathy, 2009). Internal infection caused by viruses and mycoplasma are difficult to control and are transferred by vegetative propagation. Latent pathogens especially viruses often result in the loss of plant production and a poor quality product (Phillip *et al.*, 1992). A number of factors such as high heterozygosity, polyploidy, dioecious nature hamper breeding improvement in *P. nigrum*. According to Abbasi *et al.* (2010), seed production in Black-pepper is uncertain and yields only few heterogeneous progenies due to their short viability and high sterility in post generation stages.

However, in preference to the traditional propagation method, disease and pest resistant plant sources can be obtained from germplasm. In addition, retention of good quality parameters that help production of high quality black pepper with less usage of pesticides and fungicides can be achieved by employing tissue culture technology (Krishnamoorthy and Parthasarathy, 2009). As an alternative method to the conventional vegetative propagation, *In vitro* culture offers improvements in its faster rate of clonal multiplication, allowing production of disease free, genetically stable and identical progeny (Hu and Wang, 1983; Abbasi *et al.*, 2010).

2.3 Micropropagation

Plant tissue culture is one of the major achievements of the 20th century biotechnological efforts, which currently became a well-established technology to promote production, productivity and quality (Singh and Singh, 2007). Initially, it was exploited as a research tool, primarily focused

on attempts to culture and study the development of small, isolated segments of plant tissues or isolated cells. Around the mid twentieth century, the notion that plants could be regenerated or multiplied from either callus or organ culture was widely accepted and practical applications of the plant propagation industry were ensued (Akin-Idowu *et al.*, 2009).

Plant tissue culture is a technique involving *in vitro* cultivation of plant cells and organs, which divide and regenerate into callus or particular plant organs. Plant tissue culture has revolutionized the knowledge and application of several fields of plant sciences (Cooking, 1986). Likewise, the techniques of tissue culture had played an important role in the clonal propagation, germplasm conservation and plant improvement of black-pepper (Bhat *et al.*, 1995; Sajc *et al.*, 2000).

To date, there are many reports of *in vitro* multiplication of black pepper using shoot tip explants and seedlings. The multiplication rate attained so far is around six shoots per culture in about 90 days (Bhat *et al.*, 1992). Earlier reports on micropropagation of black pepper had stated phenolic exudates from the cut surface and bacterial contamination to be serious problems hampering the culture establishment phase (Abbasi *et al.*, 2007). Methods for reducing the interferences of phenolic oxidation and systemic contamination in the course of *in vitro* cultures had been reported (Babu *et al.*, 2003; Nair and Gupta, 2006).

According to Hu and Wang (1983), micropropagation is the application of tissue culture technology for mass propagation of any economically important plant species, which offers the possibility of rapid clonal multiplication of elite plant species, allowing production of genetically stable and identical progenies. Others (George and Sherrington, 1984; Abbasi *et al.*, 2007; Gantait *et al.*, 2011) have stated it to be an alternative method to traditional propagation. It has superiority over the conventional methods of vegetative propagation, because of its potentials for high multiplication rate. However, the overall performance of these tissue cultured plants depends on the selection of the initial material (explants), provision of an aseptic environment, determination of the best media composition, growth regulators, cultivar and environmental factors, as well (Gantait *et al.*, 2011). Black pepper micropropagation had been reported using different explants from both mature and juvenile tissues (Philip *et al.*, 1992; Nazeem *et al.*, 1993; Babu *et al.*, 2003; Nazeem *et al.*, 2004; Nair and Gupta, 2005).

2.3.1 Explants

Choice of explant plays an important role in determining the efficiency of *in vitro* propagation (Abbasi *et al.*, 2007). Successful *in vitro* techniques for micropropagation of black-pepper have been reported using mature shoot-tips (Nazeem *et al.*, 1992; Philip *et al.*, 1992; Joseph *et al.*, 1996), leaf explants (Sujatha *et al.*, 2003), nodes (Bhat *et al.*, 1991), seeds (Nair and Gupta, 2006). In most of the cases, plantlets regenerated from seedling derived callus and shoot apices had been reported, though several attempts to regenerate plants from mature vine were unsuccessful (Mathews and Rao, 1984). The source of explant was important in determining the morphogenetic and regenerative potential, which were significantly influenced by the physiological conditions of the donor plant (Debergh and Maene, 1981; Read, 1988). As a result, maintaining the donor plants in a clean and controlled environmental condition could help delivery of healthy and sterile explants (Sagare *et al.*, 2001). The physiological age of explants and the explants type and size are the other factors that influence formation of organs *in vitro* (Rout *et al.*, 2000).

2.3.2 Sterilization

Plant tissue culture media, which are rich in sucrose and other organic nutrients, support the growth of many microorganisms like bacteria and fungi, once reaching on the medium these microbes generally grow much faster than the cultured tissue and finally kill it. In all cases, the explant materials should be free from dirt and debris, and washed in a mild detergent solution and rinsed several times with distilled water prior to sterilization (Kodym *et al.*, 2001).

The type and concentration of the sterilant, as well as duration of the treatment are the major factors to determine the success rate of micropropagation. The type of sterilizing agent selected should easily be removed from the surface of the explant while rinsing, and it should also be environmentally friendly (Singh and Singh, 2007). To disinfect plant tissues, varied sterilizing agents such as Sodium hypochlorite (NaOCl, at the rate of 0.025% - 0.25%), Calcium hypochlorite (CaOCl, at a rate of 9 - 10%), Hydrogen peroxide (H₂O₂ solution at a rate of 3 - 10%), and others like 1% - 2% Bromine water, 1% Silver nitrate (AgNO₃) and 0.1%-1% mercuric chloride (HgCl₂) are also used to attain the purpose (George and Sherrington, 1984). Diluted house hold bleach can also serve this purpose, which normally contains 5.25% NaOCl. It is

equally effective and less expensive (Kodym *et al.*, 2001) than the other laboratory grade sterilizing agents containing similar ingredients. After treatment with the sterilants, explants must be thoroughly rinsed with several changes of sterile distilled water. The type and concentration of chemical sterilant to be used and exposure time vary from species to species, and organ to organ or tissue to tissue, and must be decided based on experimental data (Bhojwani and Razdan, 1996; Feyissa *et al.*, 2005).

According to Philip *et al.* (1992), and Hussain *et al.* (2011), sterilizing black pepper shoot tip explants with 0.1% HgCl₂ for one minute or 0.1% NaOCl for 10-15 minute after all the leaves were removed and rinsed in distilled water, followed by three washings with sterile distilled water was stated effective where in contamination was not found to be a substantial problem. However, treatment with mercuric chloride is not commonly recommended, as such chemicals of heavy metal derivatives are highly poisonous and hazardous to human health, unless handled with great care (Deberge and Zimmerman, 1990; Feyissa *et al.*, 2005).

2.3.3 Basic media

Vasil and Thorpe (1994) had indicated the success in cell, tissue and organ culture technology to be related with the selection of culture media components. Plant tissue culture media includes all essential minerals, vitamins and a carbohydrate source, which is usually added in the form of sucrose, as well as other growth hormones (plant growth regulators). The inorganic salt formulation varies in various reported media, however, MS formulation of Murashige and Skoog (1962) is the most widely used one, with or without modifications (Herman, 1999). Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice, the specific nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with species. Therefore, selection of a specific medium composition is necessary including the specific types and concentrations of plant growth regulators (Debergh and Zimmerman, 1990).

2.3.3.1 Shoot induction and multiplication

According to Rema *et al.* (1995), a clonal propagation method has been developed for efficient multiplication of *P. nigrum* and multiple shoots were obtained from shoot tips, nodal and axillary

bud explants on MS medium supplemented with 1mg/l of IAA and 1mg/l of BA. Philip *et al.* (1992) on the other hand reported that, of the different growth regulators tested, BA alone or in combination with either IBA or AdSO₄, or both, supported initial proliferation of shoot tip explants. However, BA at a concentration of more than 5 mg/l, with or without IBA, suppressed proliferation and growth of shoots. Addition of 160 mg/l AdSO₄ to the medium enhanced shoot proliferation and yielded up to 14 shoots per explant but only about 20 % of the explants responded. The maximum numbers of axillary shoots were observed from shoot tip explants grown on medium containing 1.5 mg/l of BA without IBA. As a result, averages of 25 new shoots were produced per explant after 3-4 subcultures. The bunch of shoots were cut and separated into segments containing 3-4 shoots, and transferred to fresh medium, these clumps were further multiplied and about 15-20 shoots obtained within 6 weeks. Similarly, Hussain *et al.* (2011) showed that the shoot initiation was poor with 2.5 and 3.0 mg/l BA which indicate that higher the concentration of BA lowers the rate of organogenesis. Anand & Rao (2000) also reported that increasing the concentration of BA from 0 to 6.6 µM enhanced regeneration but further increase in the concentrations of BA suppressed number of shoot formation.

2.3.3.2 Root induction

The process of *in vitro* root initiation, development and elongation normally requires culture medium containing auxins (Azad *et al.*, 2003). The auxins that are commonly used in tissue culture medium are: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA); of these all, however, IBA and IAA are widely used for *in vitro* rooting (Bhojwani and Razdam, 1996).

According to Philip *et al.* (1992), to attain better rooting of black pepper shoots should be excised at 4.5cm height and transferred to ½ strength MS basal medium containing 1 µM Naphthalene acetic acid (NAA). Thereafter, 8-10 roots were obtained from each shoot and the plants derived from this *in vitro* culture resembled normal field grown plants. Likewise, others (Bhat *et al.*, 1995; Azad *et al.*, 2003) have also reported 2-4 roots from *in vitro* excised shoots within two weeks of transfer to a medium containing 1 µM IAA. Unlike Bhat (1995) and Azad (2003), Hussain *et al.* (2011) had shown excellent rooting on full strength MS medium augmented with 1.5 mg/l of IBA after eight days of culture.

3. MATERIAL AND METHOD

3.1. Tissue Culture Experiments

3.1.1. Stock Plant Preparation

Healthy and vigorously growing young plants of nationally released black pepper cultivar SI-3/80 (Tato) were obtained from Teppi National Spices Research Center (TNSRC) and used as a stock plant. The stock plants were transplanted in pots and kept in the green house under optimal growth condition to keep them vigorous and healthy.



Figure1. Stock plants of black pepper (*P. nigrum* L.) cultivar SI-3/80 kept in the green house.

3.1.2. Explant Preparation and Sterilization

Shoot tips (2cm) from main branch and lateral branches were excised and washed three times with tap water and commercial liquid detergent. Then after, the explants were kept under running water for 20 minutes, followed by immersion in a 0.3 % systemic fungicide solution (Kocide-101) for 10 minutes. The cleaned explants were then transferred to the laminar flow hood and immersed in 70 % ethanol for 1 min. The explants were then surface sterilized using of 15% berekina for 15 minutes. Finally, the explants were rinsed four times with sterile distilled water added with Cysteine-HCl (25mg/l).

Sterilized shoot tip explants were cut down to 0.5 to 10 mm size by trimming all the dead and chlorine affected tissues prior to culture. To cope up with the characteristic phenolic exudates of black pepper, the culture media at all stages were fortified with 25 mg/l Cysteine-HCl accompanied with frequent sub-culture (at monthly interval) of the explants onto a fresh medium.

3.1.3. Experiment One: Culture Initiation

For culture initiation, agar solidified (0.8% agar-agar) MS basal medium of half and full strength added with 3% sucrose were supplemented with different concentrations of BAP (0, 1, 2, 3 and 4 mg/l). Therefore, the experiment was carried out with a treatment combination of two strengths of MS and four concentrations of BAP in CRD design in 2x5 factorial combinations.

3.1.4. Experiment Two: Shoot Multiplication

Those explants responded to the prevailing culture conditions were transferred singly onto a shoot multiplication medium. The medium used was composed of MS basal medium fortified with different concentrations of BAP (0, 1, 2, 3 and 4mg/l) and Kinetin (0, 1.5 and 3mg/l). The experiment was arranged in a 5 x 3 factorial (i.e. for BAP and Kinetin, respectively) in CRD. After 45 days, explants with proliferating shoot clumps were divided and 5 shoots each were sub cultured on to a fresh medium of similar composition. The cultures were then maintained at $25 \pm 1^{\circ}\text{C}$ with a 12 hour photoperiod, and a light intensity of 1000-2000 lux emanating from cool white florescent lamps of 40 watt.

3.1.5. Experiment Three: Shoot Elongation and Rooting

For the subsequent stage of shoot elongation and rooting, individual shoots from *in vitro* grown black pepper plants were transferred to MS basal medium, added with 3% sucrose and four levels of IBA (0, 0.5, 1 and 1.5 mg/l) and four levels of NAA (0, 0.5, 1 and 1.5 mg/l). Therefore the experiment was laid in a 4 x 4 factorial in CRD, with the two factors being IBA and NAA.

3.2. Experimental Design and Data Analysis

All experiments were laid in a Completely Randomized Design (CRD) with factorial treatment combinations, having four replications per treatment and five explants per jar under each replicate. All the experiments were repeated two times to ensure reproducibility of the results and the average of these two were considered for statistical analyses using the SAS statistical software (Version 9.2) and ANOVA was constructed, followed by mean separation using appropriate procedures (REGWO). In all cases, probability level of 0.05 ($P \leq 0.05$) was considered for statistical significance.

4. RESULT AND DISCUSSION

4.1. Effect of MS basal media strength and different concentration of BAP on *in vitro* shoot bud induction of *P. nigrum* L., cv: 3/80.

The analysis of variance obtained from the data (Table 1) showed those different concentrations of BAP had very highly significant effect ($P < 0.0001$) on the shoot induction rate. Likewise, interaction effect of BAP with half and full strength MS basal media on rate of shoot induction was found to be very highly significant and highly significant (BAP*1/2MS* $P < 0.0001$ and BAP*MS, $P = 0.0003$) respectively.

The highest rate of shoot induction ($62.50 \pm 0.00\%$) was achieved on half MS medium supplemented with 2 mg/l BAP (Fig 2a). The second ranking shoot induction rate (52.00 ± 0.68) was recorded from full MS media containing 1 mg/l BAP (Fig. 2b). Therefore, for shoot tip explants, MS basal media containing 2 mg/l BAP added to half MS basal media was found to be optimum medium for *in vitro* shoot initiation of *P. nigrum* L. cv; 3/80. From all given treatments, the minimum rate of shoot induction was observed on full MS medium containing 4.0 mg/l BAP and full MS without any cytokinin (BAP) with respective rate of $12.50 \pm 0.80\%$ and $17.50 \pm 0.56\%$ (table 1).

Table1. Rate of shoot bud induction observed from five week culture of shoot tip explants of *P. nigrum* L. cv: 3/80 on different media formulation.

| Media type | Basal media and plant growth regulators | | Shoot bud induction |
|------------|---|------------|------------------------------|
| | MS media | BAP (mg/l) | (Mean %) \pm S.D. |
| Ia | Half | 0.00 | $22.50^{\text{gh}} \pm 1.33$ |
| Ib | Full | 0.00 | $17.50^{\text{h}} \pm 0.56$ |
| Ic | Half | 1.00 | $35.00^{\text{f}} \pm 0.56$ |
| Id | Half | 2.00 | $62.50^{\text{a}} \pm 0.00$ |
| Ie | Half | 3.00 | $47.50^{\text{c}} \pm 0.76$ |
| If | Half | 4.00 | $25.00^{\text{g}} \pm 1.45$ |
| Ig | Full | 1.00 | $52.50^{\text{b}} \pm 0.68$ |
| Ih | Full | 2.00 | $42.50^{\text{d}} \pm 0.76$ |
| Ii | Full | 3.00 | $37.50^{\text{e}} \pm 1.22$ |
| Ij | Full | 4.00 | $12.50^{\text{i}} \pm 0.80$ |

Means with the same letter in a column are not significantly different at $\alpha = 0.05$.

Shoot bud development capacity of *P. nigrum* shoot tip cultures increased with increase in concentration of BAP on half MS basal media from zero to 2 mg/l and reduced with further addition of BAP. For full MS basal media however, decline of shoot induction rate started from MS + 2 mg/l BAP and the highest rate of shoot bud induction was achieved on MS media containing 1 mg/l BAP (table 1). This could be due to the exogenous application of cytokinins which release the shoot buds from apical dominance. Addition above certain optimum amount however, could reduce shoot induction rate by inhibiting the availability of required endogenous amount of auxin for shoot initiation as cytokinins do not act alone unless together with auxins (George *et al.*, 2008; Hartman *et al.*, 2009).

The present result of shoot induction rate is in agreement with the report of Philip *et al.* (1992) which put MS + 1.5mg/l BAP as best treatment for shoot initiation from shoot tip explants of *P. nigrum*. The application of BAP above certain optimum level (>2-3mg/l) resulting relatively poor shoot bud induction rate is also in line with the report of Hussain *et al.* (2011) and Anand and Rao (2000).

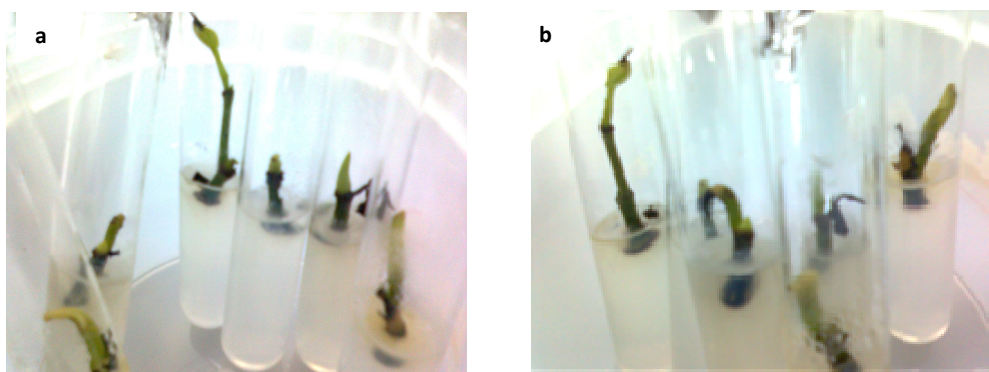


Figure 2: Axillary shoot buds developed from **a**: shoot tip explants on $\frac{1}{2}$ MS+ 2mg/l BAP after five weeks of culture. **b**: Shoot tip explants on MS + 1mg/l BAP after five weeks of culture.

4.2. Effect of different concentrations of BAP alone and together with Kinetin on *in vitro* shoot multiplication of *P. nigrum* L., cv: 3/80

Those shoot buds induced well on the prevailing shoot induction medium were transferred to $\frac{1}{2}$ MS media supplemented with BAP (0, 1, 2 and 3mg/l) alone and in combination with Kinetin (0, 1.5 and 3mg/l). Cultures were sub cultured twice and the effect of hormones on *in vitro* shoot multiplication of *P. nigrum* L., 3/80 cultivar was evaluated.

In this study, the significance of BAP alone and the interaction of BAP with kinetin were considered. The ANOVA revealed that the concentration of BAP both alone and together with kinetin had very highly significant effect ($P < 0.0001$) on shoot multiplication rate. The proliferation rate had shown progressive increase from the first sub culture to the second by an average of 25%. In this study however, further steps of sub cultures that enables the examination of the overall progress up to declining stage was not carried out. Maximum (number) of shoot proliferation (4.60 ± 0.00) was obtained on $\frac{1}{2}$ MS + 2 mg/l BAP + 1.5mg/l kinetin (Fig 3a) followed by $\frac{1}{2}$ MS + 3mg/l BAP alone which resulted 4.25 ± 0.02 shoots per explant (table 2).

Table2. Number of microshoots of *P. nigrum* L. developed (multiplied) from a shoot bud culture on different media combination after 45 days of culture.

| Media type | Plant growth regulators | | Mean No. of shoot /explant \pm S.D. |
|------------|-------------------------|-----------|---------------------------------------|
| | BAP (mg/l) | KN (mg/l) | |
| Ma | 1.00 | 0.00 | $1.50^f \pm 0.24$ |
| Mb | 2.00 | 0.00 | $3.15^b \pm 0.05$ |
| Mc | 3.00 | 0.00 | $4.25^{ab} \pm 0.02$ |
| Md | 4.00 | 0.00 | $2.12^c \pm 0.66$ |
| Me | 1.00 | 1.50 | $3.10^b \pm 1.22$ |
| Mf | 1.00 | 3.00 | $2.45^d \pm 0.43$ |
| Mg | 2.00 | 1.50 | $4.60^a \pm 0.00$ |
| Mh | 2.00 | 3.00 | $2.10^e \pm 0.12$ |
| Mi | 3.00 | 1.50 | $2.85^c \pm 0.15$ |
| Mj | 3.00 | 3.00 | $1.75^f \pm 0.10$ |
| Mk | 4.00 | 1.50 | $1.25^g \pm 0.43$ |
| Ml | 4.00 | 3.00 | 0.00 ± 0.00 |
| control | 0.00 | 0.00 | 0.00 |

Means with the same letter in a column are not significantly different at $\alpha = 0.05$.

An increase in number of shoots per shoot bud culture with increased concentration of BAP from zero up to 3mg/l might be due to the effect of cytokinin in releasing lateral buds from dormancy or breaking apical dominance by inhibiting the level of endophytic auxins. Cultures on above 3mg/l BAP had shown a decline in rate of proliferation and stunted morphology.

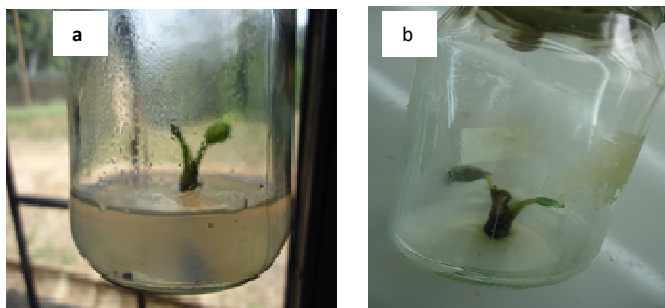


Figure 3: Microcuttings of *P. nigrum* L developed from **a**: shoot tip explants on $\frac{1}{2}$ MS+ 2mg/l BAP + 1.5mg/l KN after 5 weeks of culture. **b**: on $\frac{1}{2}$ MS + 3mg/l BAP after 5 weeks of culture.

The present result is in accordance with Philip *et al.*, (1992) which reported average proliferation of 25 new shoots per shoot bud after 4 subcultures on MS + 1.5mg/l BAP.. as well as Azad *et al.*, (2003) that noted 2-3 mg/l BAP in combination to be optimum for shoot proliferation of *P. nigrum*. In this study, however use of BAP in combination with kinetin, i.e. ½ MS + 2mg/l BAP + 1.5mg/l kinetin, in media three (multiplication media) has shown good result although BAP alone (3mg/l) is also equivalently important.

4.3. Effect of different concentrations of IBA and NAA on *in vitro* root initiation and shoot elongation of *P. nigrum* L., cv: 3/80 microcuttings.

The ANOVA had indicated a very highly significant effect, ($P < 0.0001$) of ½ MS strength with IBA and NAA for *in vitro* shoot elongation and rooting of *P. nigrum* L., cv: 3/80 microcuttings. In this result both IBA and NAA were found to be effective in promoting root induction as well as shoot elongation at 1 mg/l concentration in half strength MS basal medium. More than 75 % of microcuttings were rooted after four weeks of culture. The highest number of roots (8.20 ± 0.00), root length (6.33 ± 0.10 cm) and shoot length (4.45 ± 0.75 cm) was recorded from half MS medium containing 1mg/l IBA followed by half MS medium containing 1mg/l NAA resulting in 6.41 ± 0.05 root number, 6.10 ± 0.00 cm root length and 3.52 ± 0.75 cm shoot length (Table 3). The roots formed were normal longer thicker and with many thin branches.

Table3. Shoot elongation and rooting of *P. nigrum* microcuttings after five weeks of culture.

| Media type | Plant growth regulators | | Shoot length | Root | Root length |
|------------|-------------------------|------------|----------------------|-------------------|--------------------|
| | IBA (mg/l) | NAA (mg/l) | Mean (cm) \pm S.D. | Mean \pm S.D | Mean(cm) \pm S.D |
| Era | 0.50 | 0.00 | $3.33^{cd} \pm 0.00$ | $5.34^c \pm 0.07$ | $3.75^c \pm 0.06$ |
| ERb | 1.00 | 0.00 | $4.45^a \pm 0.75$ | $8.20^a \pm 0.00$ | $6.33^a \pm 0.10$ |
| ERc | 1.50 | 0.00 | $3.82^b \pm 0.21$ | $4.66^d \pm 0.63$ | $4.50^b \pm 0.71$ |
| ERd | 0.00 | 0.50 | $2.85^d \pm 0.00$ | $5.10^c \pm 0.00$ | $3.25^d \pm 0.34$ |
| ERe | 0.00 | 1.00 | $3.52^c \pm 0.75$ | $6.41^b \pm 0.05$ | $6.10^a \pm 0.00$ |
| ERf | 0.00 | 1.50 | $3.33^{cd} \pm 0.04$ | $3.65^e \pm 0.42$ | $4.20^b \pm 0.00$ |
| control | 0.00 | 0.00 | $1.76^d \pm 0.04$ | 0.00 | 0.00 |

Means with the same letter in a column are not significantly different at $\alpha = 0.05$.

This result is in agreement with Philip *et al.* (1992), who reported better rooting (8-10 roots) of black pepper shoots on ½ MS containing 1 μ M NAA in that 8-10 roots were obtained from each shoot. Bhat *et al.*, (1995) and Azad *et al.*, (2003) have also reported 2-4 roots from *in vitro* excised shoots within two weeks of transfer to a medium containing 1 μ M IAA.

The present result however had shown reduction in rooting with increased concentration of both IBA and NAA from 1 to 1.5 mg/l. This may be due to the high concentration of auxin in the range that normally stimulates elongation of shoots (10^{-5} to 10^{-6} M), cause a significant inhibition of root growth. The inhibition of root growth and development might partly be due to the ethylene production which is triggered by high auxin concentration (Hopkins and Huner, 2004).

5. CONCLUSION

In the present study, by producing multiple microshoots (four up to five per explant/culture) from shoot tip explants followed by elongation and rooting, it has been made possible to propagate an average of 27 plantlets of 3/80 cultivar of *P. nigrum* L. from single explant in a single sub culture after the initial transfer on multiplication medium. This technique, therefore, presents an efficient system of *in vitro* clonal propagation compared to the conventional propagation method for rapid multiplication, production of a relatively disease free plants, non-seasonal production and germplasm conservation. Summary of results obtained from the present experiments are given below:

- The maximum percentage of shoot induction ($62.50 \pm 0.00\%$) was observed on half MS medium supplemented with 2mg/l BAP from shoot tip explants of *P. nigrum* L., cv: 3/80
- Therefore, Half MS basal media containing 2mg/l BAP was found to be optimum media for *in vitro* shoot initiation of *P. nigrum* L., cv: 3/80
- Half MS basal medium supplemented with 2mg/l BAP and 1.5 mg/l KN resulted in $4.60 + 0.00$ shoot number with best vigor and morphological appearance.
- Half MS containing 3mg/l BAP alone also produced ($4.25^{ab} \pm 0.02$) shoot number with good morphological appearance.
- The number of microshoots per shoot bud/explant increased from the first sub culture to the second by 25 %.
- Best rooting and shoot length was achieved on half strength MS basal media containing 1 mg/l IBA which resulted mean values of 8.20 ± 0.00 root number, 6.33 ± 0.10 cm root length and 4.45 ± 0.75 shoot length followed by half strength MS basal media containing 1 mg/l NAA with 6.41 ± 0.05 root number, 6.10 ± 0.00 cm root length and 3.52 ± 0.75 cm shoot length.

Thus, this protocol could be useful for large-scale production of highly demanded 3/80 cultivar of *P. nigrum* L. true-to-type and provide a possible system towards genetic improvement of the crop using shoot tip explants sources.

6. RECOMMENDATIONS

- ✓ This protocol could aid valuable input for further investigation and it has to be fully optimized to be used widely for the given genotype and adopted for protocol development and optimization of other related cultivars.
- ✓ Field performance evaluation and comparison for quantity and quality of active ingredient (Piperine) with the *ex vitro* grown parent plants should be carried out in order to actually measure the overall advantage of tissue culture over that of conventional propagation for the given cultivar.

7. ACKNOWLEDGEMENT

I acknowledge Adama Science and Technology University (ASTU) for funding all the expenses required to carry out this research project. Jimma Agricultural Research Center (JARC) is also highly appreciated for allowing us their Biotechnology (tissue culture) laboratory where in the experiments included in this study was performed.

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FINAL REPORT SUBMISSION FORMAT

I. I CERTIFY THAT THE INFORMATION AND FIGURES GIVEN IN THE REPORT ARE CORRECT AND COMPLETE TO THE BEST OF MY KNOWLEDGE.

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

DATE

II. APPROVED BY THE HEAD OF THE DEPARTMENT

DATE

NAME & SIGNATURE: _____

III. APPROVED BY THE SCHOOL/ASSO. DEAN FOR RPG

DATE

NAME & SIGNATURE: _____

FOR RPO USE