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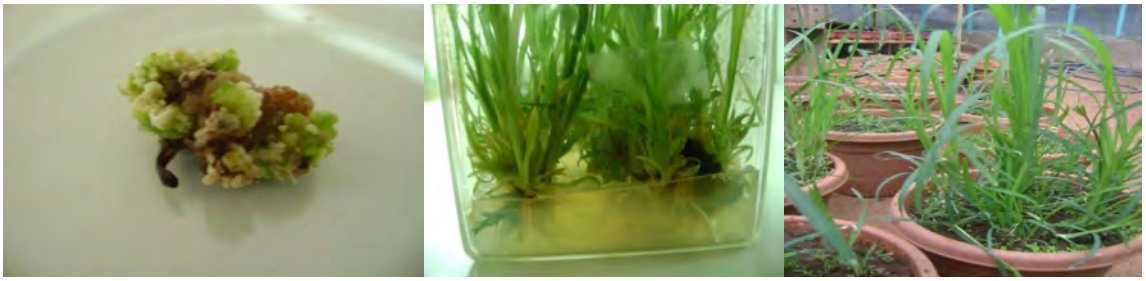
Somatic Embryogenesis of Finger Millet (*Eleusine coracana* (L.) Gaertn) using seed explants



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Abstract

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*Finger millet (*Eleusine coracana *subsp. coracana) is one of the most important crops in Africa and India. Finger millet contains different nutrients and is a popular food among diabetic patients in different countries. Finger millet is an orphan crop of developing world on which little research has been done. The improvement of this crop is crucial towards abiotic and biotic stress tolerance. Tissue culture and transformation techniques are among the approaches to improve this crop. Therefore, the objective of this study is to develop an efficient somatic embryogenesis protocol for regeneration of different finger millet cultivars. Somatic embryogenesis protocol for regeneration of four cultivars of finger millet is developed. For all cultivars, mature seeds were used for callus induction. After sterilization of the seeds, callus was induced efficiently in different concentrations of 2, 4-D in all cultivars. From 95 to100% callus induction were recorded on callus induction medium containing 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l 2, 4-D. MS-medium supplemented with 1mg/l 2, 4-D resulted in 45 to 58.3% somatic embryogenesis and 1mg/l KN in combination with 0.25mg/l NAA was resulted with highest mean number (*15.85±0.63) *of shoots and* 16.74±1.86 *roots were recorded from* AAUFM-19.

Key Words: callus induction, Kinetin, NAA, shoot regeneration, somatic embryogenesis

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List of Abbreviations

ANOVA Analysis of variance

2, 4-D 2, 4-Dichlorophenoxyacetic acid

NAA α-Naphthalene acetic acid

KN kinetin

SPSS Statistical package for social science

SE Standard error

1. Introduction

The term “millet” is given to different warm-season annual grass crops around the world that are harvested as grain for human food or animal feed. Millets are similar to sorghum forage in their productivity and feed value. There are several advantages of millets more than sorghum when grown for forage, including no prussic acid potential. In addition to this; they are tolerant soil with high pH conditions. Millets are generally considered as negligible crops in another place, but in India, Africa, and China they have a great importance and solving hunger problem of many people in those countries. When compared to other cereal grains, millets can grow well on less fertile soils and non-suitable growing conditions (Mark *et al.,* 2012), such as in dry areas, temperate, subtropical and tropical regions (Baker, 1996). In addition, their rapid growth and shorter growing seasons make millets the best for emergency and binary cropping conditions (Mark *et al.,* 2012). Millets are the third most important cereal crops in Africa after maize and sorghum. They are grown in the harsh semi-arid tropics of Africa where inadequate rainfall and lack of irrigation make production of other cereal crops difficult to sustain. A general impression is that research to improve millets has generally lagged worldwide because they are not grown as food crops in the developed world and in Africa, they are considered as "poor man’s crops."(Mywish *et al.,*1998).

The millets consist of five genera of the Panaceae family (*Panicum, Setaria, Echinochloa, Pennesetum* and *Eleusine).* The most important cultivated species are: Proso millet (*Panicum miliaceum*), Foxtail millet (*Setariaitalica*), Japanese barnyard millet (*Echinochloafrumentacea*), Finger millet (*Eleusine coracana*) and Kodo millet (*Paspalum scrobiculatum*)(Pragya and Rita, 2012). Finger-millet (*Eleusine coracana)* gains its name from the head of the plant, which bears

some similarity to splayed hand (Roger, 2012). Finger millet, native to East Africa, is entwined in the local culture and traditions. However, in spite of its importance to the livelihoods of millions of small-holder farmers in East Africa, its valuable nutritional and processing properties, the growing demand exceeding supply, and its regional and international trade potential, finger millet has largely been neglected by national and international research organizations and major donors to agricultural research in sub-Saharan Africa. This neglect has contributed to a lack of realization of the potential productivity of finger millet. Increased production, utilization and trade of finger millet in East Africa are currently limited by a number of constraints (Mgonja *et al*.,2005).Crop damage by insects is minimal but pests such as birds and *Striga* weed are a constant serious threat to the crop (Esele 1989). The most serious biotic constraint is the blast disease caused by the fungus *Magnaporthe grisea*. Blast affects finger millet at all growth stages, particularly causing major losses through neck and Panicle infections (Mgonja et al., 2005).Finger millet blast disease is by far the most devastating, causing over 50% yield loss (Esele 1989). Other constraints to finger millet production include poor incentives and marketing arrangements – low pricing, poor and inaccessible market channels, inaccessibility to credit facilities and inadequate improved processing and product development facilities at commercial levels (Mgonja *et al*., 2005). To meet the strong increase in cereal demand worldwide, new approaches and technologies for generating new varieties are necessary. One of these methods is the creation of transgenic plants with desirable traits. Although millets are economically important, especially in the developing world, little genetic improvement has been done so far specifically using wide- or cross- hybridization among closely related species. The incompatibilities due to inter specific hybridization are alleviated by directly transferring the desirable traits to millets using optimum or efficient transformation method. Hence, crossing

barriers could be overcome, and genes from unrelated sources would be introduced asexually into crop plants. Monocots in general and cereals in specific were initially difficult to genetically engineer, mainly due to their recalcitrance to in vitro regeneration and their resistance to *Agrobacterium*-mediated infection. However, efficient transformation protocols have been later established for the major cereals including rice and maize. Gene transfer to millets would be facilitated once efficient or optimum regeneration has been developed (Plaza-Wüthrich and ZerihunTadele, 2012). The objective of this study is to develop somatic embryogenesis protocol for finger millet (*Eleucinecoracana*).

2. Literature review

2.1. Description and taxonomy of finger millet (*Eleusine coracana*)

Finger millet (*Eleusine coracana* subsp. *coracana*) and its wild relatives are the members of *Chloridoidea*, one of the primary subfamilies of the grass (*Poaceae*) family. The cultivated *E.coracana* is a tetraploid species (2n=4x=36) derived from its wild ancestor *E. coracana* subspecies *africana* (Zeven and de Wet, 1982 as cited in Kebere et *al.*, 2006). It is highly self-pollinated and has 36 or 72 (2N) chromosomes (Rachie, 2011).Finger millet has different common names in different countries such as ragi and mandua (India); koddo (Nepal); finger horse (Germany); petit mil, eleusine cultivee, coracan, koracan (France); bulo (Uganda); kambale, lupoko, mawele, amale, bule (Zambia); poho, rapoko, zviyo, njera, mazhovole (Zimbabwe); finger millet, African millet, koracan (England); dagussa, tokuso, barankiya (Ethiopia); wimbi, mugimbi (Kenya). It is an important staple food in parts of eastern and central Africa and India (Yilma Kebede and Abebe Menkir, 1986).

2.2. Morphological structure of Finger millet

Finger millet is a feathered annual crop, growing to a height of 30–150 cm and is harvested in 75–160 days. Leaves are narrow, grass-like and able to produce many tillers and nodal branches. The panicle consists of a group of digitally arranged spikes often referred to as fingers. The spikelets are made up of four to ten florets arranged on the finger. All florets are perfect flowers with the exception of the terminal ones which may sometimes be infertile. The grain is oblong to round and oval shape, reddish brown, creamy white, and dark brown in color with the grains' surface finely roughened. The grain of finger millet has a good aroma when cooked or roasted

and is known to have many health promoting qualities. The grain can be stored for years without insect damage, which makes it a particularly valuable crop for famine facing areas (Prem, 2012).

2.3. Origin and distribution of Finger millet

East Africa is recently reported as the center of origin and diversity of finger millet (FAO, 1998). Finger millet is widely recognized that the highlands of Africa, especially Ethiopia/Uganda, are the primary center of origin and the Indian sub-continent is the secondary center. The crop has been in cultivation on both continents for more than 5000 years, but separated both morphologically and genetically. Wild finger millet (subspecies *africana*) is native to Africa but has migrated to several warmer parts of Asia and America (Prem, 2012).The crop was one of the dominant crops in the late Aksumite period (100 BC to 300 AD). Further, it has been noted that the late Aksumite populations were mainly occupied in producing and processing food and that the range of cultivated crops including finger millet was remarkably similar to that exploited in the region during more recent times. Finger millet has an old history of domestication in Ethiopia, particularly in the northern part of the country. The crop has been under cultivation since the time immemorial. The values and the strong relation that the crop has to the livelihood of the people in the Tigray region of Ethiopia expresses as traditional songs, sayings and poems that are transferred from generation to generation through oral traditions and continue to expand biological adaptation and use value attributes of the crop (National Research Council, 1996).

2.4. Cultivation of Finger millet

Finger millet cultivation is more widespread in terms of its geographical adaptation compared to other millets. It has the ability to survive different conditions of heat, drought, humidity and tropical weather. It is an important staple in many parts of eastern and southern Africa, as well as in South Asia. Besides this, the precise estimate of area and production at the global level is not available except for India and Africa. The global annual planting area of finger millet is estimated at around 4-4.5 million hectares, with a total production of 5 million tons of grains, of which India alone produces about 2.2 million tons and Africa about 2 million tons. The rest comes from other countries in South Asia. The important finger millet growing countries in eastern and southern Africa have been especially the sub-humid regions of Ethiopia, Kenya, Malawi, Tanzania, Uganda, Zaire, Zambia and Zimbabwe. Similarly, in South Asia the crop is largely grown in India, Nepal and, to some extent, in Bhutan and Sri Lanka. In addition to this, finger millet is reported to be grown in both China and Japan to a limited extent (Prem, 2012). Finger millet is often cultivated in semi-arid and arid agro ecology, where it is frequently affected by drought (Masresha Fetene *et al*., 2011). It is extensively cultivated in the tropical and sub-tropical regions of Africa and India is known to save the lives of poor farmers from starvation at times of extreme drought (Dagnachew Lule *et al*., 2012). It is indigenous to Ethiopia and occupies 304,758 ha of land with production of 305,101 tons (CSA, 2008). It is grown more or less throughout the country though mainly cultivated in the mid and low altitude of regions of Gonder, Gojam, Wollega and Tigray where it constitutes 10 to 20% of the total cereal production (Yilma Kebede and Abebe Menkir, 1986). The yields of finger millet are low in Ethiopia due to different production problems including: lack of improved varieties, little research emphasis given to the crop, non-adoption of improved technologies, poor attitude to the

crop, disease like blast which is the most serious disease, lodging and moisture stress in dry areas, threshing and milling problem are some of the most serious production constraints in finger millet production in Ethiopia (Yemane Tsehaye and Fassil Kebebew, 2002; Kebere Bezaweletaw *et al.,* 2006; Erenso Degu *et al*., 2009; Andualem Wolie, 2009). Similarly in South India, it is the fourth cereal after rice, wheat and maize. Finger millet is grown in highly advanced situation. The yields of this crop are very low because the crop suffers from drought very frequently during its growth period. The other problems faced by this crop are blast diseases and downy mildew. Both are fungal diseases. Hence, the main stresses that the crop suffers are drought and blast. This can become every severe and the losses are sometimes up to 60%. Leaf blast is not very common but neck and finger blast are common and cause a risk to maximum yield loss (Mark *et al.,* 2005).

The major attributes of finger millet are its adaptability to adverse agro-ecological conditions with minimal inputs, tolerant to moisture stress, produced on marginal land where other crops cannot perform and tolerant to acidic soil and termites (Barbeau, and Hilu, 1993). Furthermore, it has high nutritional value and excellent storage qualities (Dida, 2007). Therefore, finger millets represent one of the critical plant genetics resources for the agriculture and food security of poor farmers that inhibit arid, infertile and marginal lands (Dagnachew Lule *et al.*, 2012).

Finger millet has a relatively wide range of adaptation within moderate temperatures and moisture ranges. It is most widely cultivated on hilly, lateritic soils in the 500-1000mm rain fall belt of the tropics and subtropical regions. It has high yielding potential producing highest mean yield among the millets in Africa and India, and is frequently grown both dry and irrigated on lands where moisture is insufficient for other crops. The grain is very nutritious, and has malting properties (Rachie, 2011).

2.5. Importance of finger millet

Finger millet is used to make several dishes and drinks, particularly in the rural areas. The non-distilled local beer (Tella) and the porridge made of the crop are believed to cure diseases such as diarrhea and malaria, and assist healing of fractured bones. Even though Tella can be prepared from several crops such as sorghum, maize and barley, Tella used at wedding ceremonies and other religious festivals must be made of finger millet, in central and western Tigray. Tella is also essential for labor group workers, at weeding and harvesting. The Muslims also prepare non-alcoholic local drinks (karibo) made from finger millet. The traditional drinks Tella and areki (distilled liquor) also serve as an income source in small towns and in some parts of the rural areas. Women farmers are usually involved in such kind of activities to complement income of the household. The unleavened bread (kita) and the residual left after the purified Tella taken away is also used to feed animals. In the rural areas, finger millet is consumed as enjera which is tasty and easily digested (National Research Council, 1996).

Finger millet provides food grain as well as straw which are appreciated animal feed, especially in the rain fed areas. Among the major food grains, finger millet is one of the most nutritious crops for protein, minerals (calcium and iron) and amino acids (methionine, an amino acid lacking in the diets of hundreds of millions of the poor who live on starchy foods such as cassava, plantain, polished rice, and maize meal); and provides 8-10 times more calcium than wheat or rice. Finger millet carbohydrates are reported to have the unique property of slower digestibility and can be regarded as food for long sustenance. The excellent malting qualities have added to the uniqueness of the grain in expanding its utility range in food processing and value addition. Finally, the crop is productive in a wide range of environments and growing conditions, from southern Karnataka state in India to the foothills of the Himalayas in Nepal, and

throughout the middle elevation areas of Eastern and Southern Africa (Prem, 2012). Finger millet is also the most important of the small millets grown for food. It is an important food crop in South Asia and Africa. It is nutritionally comparable or even superior to major cereals in respect of protective and useful nutrient content. It is rich in calcium and has good amount of magnesium, phosphorus and iron. Finger millet protein has a favorable amino acid variety that includes cystine, tyrosine, tryptophan and methionine (Rachie, 1975).

Nutritionally, finger millet is good source of different nutrients, minerals and fiber. Total carbohydrate content of finger millet has been reported to be in the range of 72 to 79.5%. About 80 to 85% of the finger millet starch is amylopectin and remaining 15 to 20% is amylose non-starch polysaccharide account for 20 to 30% of the total carbohydrates in finger millets. Reducing sugar in the range of 1.2 to 1.8% and 0.03% non-reducing sugar is found in finger millet. The *in vitro* starch digestibility of native finger millet is 71.67%. Total dietary fiber insoluble dietary fiber and soluble dietary fiber content in finger millet is 12, 11 and 2%, respectively. Finger millets have hypoglycemic effect, which is attributed to high fiber content. High fiber diets containing complex carbohydrates are slowly digested and absorbed, thus bring reduction in postprandial glucose. The second major component of finger millet is protein. It has nearly 7% protein but large variations in protein content from 5.6 to 12.70% have been reported (Pragya and Rita, 2012). Finger millet is a popular food among diabetic patients in different countries. Its slow digestion indicates low blood sugar levels after a finger millet diet thereby reacting as a safer food for diabetics. It has also anti oxidant and antimicrobial properties (Palanisamy *et al*., 2011). In spite of all these advantages, finger millet has been a neglected crop, both at national as well as global levels. The crop is little known in many countries in Europe, or South and North America. In recent years, the production of this neglected crop has

been declining rapidly, airing fears that finger millet grain may become a rare commodity (Prem, 2012). Thus in vitro culture has a unique role in sustainable and competitive agriculture and forestry, and has been successfully applied in plant breeding, and for the rapid introduction of improved plants. This becomes true through plant tissue culture technology.

2.6. Plant Tissue Culture and its Advantage

Plant tissue culture is the production of plant cells, tissues, or organs on specially formulated nutrient media under aseptic environment and controlled conditions of light, temperature, and humidity. Under the right conditions, an entire plant can be regenerated from a single cell. Plant tissue culture is a technique that has been about more than 50 years. There are several types of tissue culture depending on the part of the plant (explants) used (Evans *et al*., 2003).The development of plant tissue culture as a fundamental science was closely linked with the discovery and characterization of plant hormones, and has facilitated our understanding of plant growth and development. Furthermore, the ability to grow plant cells and tissues in culture and to control their development forms the basis of many practical applications in agriculture, horticulture industrial chemistry and is a prerequisite for plant genetic engineering (Fowler, 1987). The green revolution is considered to have resulted, at least in part, from the application of Mendelian genetics to crop improvement. This has resulted in the maximization of yields of many crops grown under conditions that reduce insect and disease pressure and on soils enriched with inorganic fertilizer. During the 1960s, it was realized that the production gains of the green revolution would be overcome by world population increases within a few decades. Therefore, the development of alternate strategies for increasing plant productivity was considered to be essential. *In vitro* procedures for manipulating plant differentiation growth and development, including production of haploid plants from cultured anthers, regeneration of plants from cell

cultures, and protoplast isolation, culture and fusion of haploids were considered to be essential parts of this new technology. Cell culture coupled with molecular biology for crop improvement has been referred to as the 'genetic engineering revolution' (Wagramer, 2004).

Plant tissue and cell cultures are usually initiated from pieces of whole plants. During the past few decades tissue culture techniques have been developed that could be used for the improvement of crop plants. Comparatively, monocotyledons are regarded as difficult *in vitro* material. The potential value of cell, tissue culture as tool for use in the improvement of crop plants has been described (Green 1977; Vasil, 1987).

Tissue culture technology is used for the production of doubled haploids, cryopreservation, propagating new plant varieties, conserving rare and endangered plants, difficult to propagate plants, and to produce secondary metabolites and transgenic plants. The production of high quality planting material of crop plants and fruit trees, propagated from vegetative parts, has created new opportunities in global trading, benefited growers, farmers, and garden owners, and improved rural employment. However, there are still major opportunities to produce and distribute high quality planting material, e.g. crops like banana, date palm, cassava, pineapple, plantain, potato, sugarcane, sweet potato, yams, ornamentals, fruit and forest trees. The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year round basis under disease free conditions anywhere irrespective of the season and weather. However, the technology needs high capital, labor and energy investment. Although, labor is cheap in many developing countries, the resources of trained personnel and equipment are often not readily available. In addition, energy, particularly electricity, and clean water are costly. The energy requirements for tissue culture technology depend on day temperature, day length and relative humidity, and they have to be controlled

during the process of propagation. Individual plant species also differ in their growth requirements. Hence, it is necessary to have low cost options for weaning, hardening of micro propagated plants and finally growing them in the field.

Plant tissue culture techniques have a vast potential to produce plants of superior quality, but this potential has been not fully exploited in the developing countries. During *in vitro* growth, plants can also be primed for optimal performance after transfer to soil. In most cases, tissue cultured plants outperform those propagated traditionally. Thus *in vitro* culture has a unique role in sustainable and competitive agriculture and forestry, and has been successfully applied in plant breeding, and for the rapid introduction of improved plant. The improved resistance to diseases and pests enables growers to reduce or eliminate the application of chemicals (Wagramer, 2004).

2.7. Regeneration and somatic embryogenesis

Regeneration of whole plants from callus cultures occurs in two general path ways, via organogenesis and embryogenesis. The later gives rise to most plants of unicellular origin and production of clonal plants, enabling crop improvement by means of tissue culture to become more effective. Regeneration via somatic embryogenesis of major cereal crops including corn, rice, sorghum, and wheat has been reported, but embryogenesis and regeneration from callus cultures have been inconsistent in cereals. Thus an observable challenge for cereal tissue culture workers is to systematically develop *in vitro* technologies for faster and more expected production of embryogenesis and plant regeneration (Guanglai, 1988).The capability of gametophyte cells to form *in vitro* embryos which are competent to develop or to regenerate is a particular characteristic of plants. In this consideration, microspore and somatic embryogenesis can be regarded as model system to investigate the mechanisms of plant embryogenesis and development, and the whole process of plant cell differentiation. The fact that the embryos can

develop from microspore or somatic cells also demonstrate the genetic program for embryogenesis can be completed outside of sexual reproduction (Henry *et al*., 1994).

Current progress in plant genetics and biotechnology is highly dependent on the use of *in vitro* cultures. Hence the establishment of effective *in vitro* plant regeneration systems enabling a rapid production of fertile, genetically 'solid' plants is of great interest to plant biotechnologists. Among the various *in vitro* systems applied, somatic embryogenesis (SE) is of special importance. It offers opportunities for *in vitro* production of true to type plants by clonal propagation as well as regeneration of genetically modified plants by genetic transformation, and somatic hybridization and *in vitro* mutant induction and selection. Moreover, SE is a useful tool in basic research on totpotency and on the fundamental processes of plant morphogenesis. Thus, the possible broad applications of SE in both basic and applied research have stimulated studies on the determination of *in vitro* conditions for the induction of somatic embryos, and their further development into complete plants. According to this, an increasing number of protocols describing efficient *in vitro* systems based on regeneration via SE are being published. Remarkable progress in the development of *in vitro* systems is enabling induction of SE in many economically important plants, as well as in model species. In recent years efficient protocols on SE induction and plant regeneration have been accessible also in *Arabidopsis thaliana* (L.) Heynh, a model organism in plant genetics and embryogenesis (Malgorzata, 2004). One of the most important prerequisites for genetic manipulation of plants *in vitro* has been the ability to grow somatic cells in sterile plant growth medium and to regenerate plants from these cultures(Litz and Gray,1995).Somatic embryogenesis is developmental process by which somatic cells undergo reorganization to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of somatic or non-

zygotic embryo capable of regenerating plants. Somatic embryogenesis represents a unique developmental pathway that includes a number of characteristic events: dedifferentiation of cells, activation of cell division and reprogramming of their physiology, metabolism and gene expression patterns (Yang *et al*., 2011 cited in Miroslava, 2013). Somatic embryos can develop indirectly, through callus tissue (ISE) or directly from explants tissue (DSE).Somatic embryos developing via direct somatic embryogenesis are formed from competent cells of explants, contrary to indirect somatic embryogenesis, are able to undergo embryogenesis without dedifferentiation or callus formation. It is believed that both processes are extremes of one continuous developmental pathway (Carman, 1990). Distinguishing between direct somatic embryogenesis and indirect somatic embryogenesis can be difficult (Emons, 1994) and both processes have been observed to occur simultaneously in the same tissue culture conditions (Turgut *et al*., 1998).

Direct somatic embryogenesis called primary somatic embryogenesis, and somatic embryogenesis through callus is called secondary somatic embryogenesis in the culture of somatic embryos. A much higher efficiency of secondary somatic embryogenesis over primary somatic embryogenesis has been indicated for many plant species (Raemakers *et al*., 1995; Akula *et al*., 2000; Vasic *et al*., 2001). Some cultures are able to retain their competence for secondary embryogenesis for many years and thus provide useful material for various studies, as described for *Vitisrupestris* (Martinelli *et al*., 2001).

Somatic embryogenesis on culture of ‘embryonic’ explants is the most common method and regular feature of plant regeneration in all the major species of cereals and grasses (Park and Walton, 1989; Vasil, 1988).

Genetic improvement of the crop depends on the combined manipulation of tissue culture techniques (Das and Misra, 2010). Production of transgenic plants with desired qualities is possible by genetic transformation of the desired genes in to the selected plants through the methodology of tissue culture. Efficient callus formation and regeneration is an important requisite to perform *Agrobacterium*-mediated transformations for producing transgenic plants (Anjaneyulu *et al.,* 2012). *Agrobacterium tumefaciens*-mediated genetic transformation has been successfully demonstrated with a wide range of important crop species (Litz and Gray, 1995). One of the most important prerequisites for genetic manipulation of plants *in vitro* has been the ability to grow somatic cells in sterile plant growth medium and to regenerate plants from these cultures (Christianson, 1987).Theoretically, the regenerants are derived from single, totipotent cells and this has been demonstrated with several species. However, under certain growth conditions (and particularly with organogenesis), morphogenesis can involve more than one cell (Christianson, 1987). It is generally considered that somatic embryos are derived either from single cells or from single cells within a pro embryonic mass. Somatic embryogenesis, therefore, is a more efficient pathway for studies involving production of genetically transformed plants.

3. Objectives

3.1 General objective

- ✓ To develop somatic embryogenesis protocol for finger millet (*Eleucine coracana*)

3.2 Specific objectives

- ✓ To determine seed sterilization protocol in finger millet
- ✓ To induce callus from seeds of finger millet
- ✓ To optimize concentrations of plant growth regulators for embryogenic callus induction in different cultivars of finger millet
- ✓ To determine plant growth regulators for regeneration of plants from somatic embryos of different cultivars of finger millet
- ✓ To acclimatize *in vitro* rooted finger millet cultivars

4. Materials and methods

4.1. Plant material

Mature seeds of four cultivars of finger millet, AAUFM-19, AAUFM-14, AAUFM-10 and AAUFM-20 were obtained from Addis Ababa University Bio-innovative project which are collected from different localities of Tigray region. Out of these, AAUFM-14 is drought susceptible, and the rest three are drought resistant.

4.2. Stock Solutions and Media preparation

4.2.1 Preparation of macronutrient, micronutrient and vitamin stock solutions

All of MS-stock solutions were prepared in different compositions and concentrations of chemicals. For macro nutrient stock solution, KNO_3 38 g/l, NH_4NO_3 33 g/l, $CaCl_2.2H_2O$ 8.8 g/l, $MgSO_4$ 7.4 g/l and KH_2PO_4 3.4 g/l were added together in to one liter flask and stirred until the contents became dissolved and poured in to plastic bottles and stored at -20 °C(Appendix 1).

4.2.2 Preparation of stock growth regulators

Growth regulators 2, 4-Dichlorophenoxyacetic acid (2, 4-D), α-naphthalene acetic acid (NAA) and kinetin (KN) 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. The powder was first dissolved in 3-4 drops of 1M NaOH or HCl and stirred followed by adding double distilled water and stirred by using magnetic stirrer. Finally, the stock solutions were stored at 4 ºC for immediate use.

4.2.3 Culture Media preparation

MS stock solutions (100 ml/l macro, 10ml/l micro including 10ml/l iron-EDTA and 10 ml/l vitamin) and different concentrations and combinations of plant growth regulators and 30g/l sucrose were used to prepare culture medium in different stages. For callus culture, MS medium supplemented with different concentrations of 2, 4-D (1.0, 1.5, 2.0, 2.5, 3.0 mg/l) was prepared. First 30 g sucrose and MS stock solutions in the above amount was added into1Lvolumetric flask and mixed by using magnetic stirrer and the pH was adjusted to 5.8 using 1M HCl and/or 1M NaOH.

After adding 8 g/l agarose, the medium was boiled in microwave oven and autoclaved at 121 ^{o}C for 15 minutes. Then 20 ml medium was poured in to 90 mm diameter sterile Petri dishes. For somatic embryogenesis, MS- medium containing 1 mg/l 2, 4-D was used.

MS- medium supplemented with 1 mg/l KN and 0.25 mg/l NAA was used for regeneration from somatic embryos. All the components of MS-medium were added to volumetric flask and stirred by magnetic stirrer then transferred to beaker and boiled by using microwave oven in order to melt the agar and 50 ml was poured in to Magentas GA-7 culture vessel followed by autoclaving at 121^{o}C for 15 minutes and kept at 4^{o}Cto be used the next day.

4.3. Seed Sterilization

About 400 to 500 mature seeds of four finger millet cultivars were washed under tap water using Ariel powder detergent and rinsed with distilled water. The seeds were then washed in 70 % ethanol for 1 min and rinsed three times with sterile distilled water followed by sterilizing in 20 % Clorox containing a drop of Tween-20 for 15 min. The seeds were rinsed five times with sterile distilled water.

4.4. Callus Induction

Twenty sterilized seeds were cultured per Petri dish in three replications on callus induction MS medium containing different concentrations of 2-4, D (1 mg/l, 1.5 mg/l, 2 mg/l, 2.5 mg/l and 3 mg/l). For each cultivar and treatment, a total of 60 seeds were used and kept under darkness at 24 ºC for four weeks. The four-week-old calli were transferred to the same fresh callus induction medium and kept under darkness at 24 ºC for additional four weeks and results were recorded. Growth regulators-free MS medium was used as control in all experiments.

4.5. Somatic embryogenesis

All eight-week-old calli were transferred to embryo induction MS medium containing 1 mg/l 2, 4-D and maintained at light intensity of 20 µmol $m^{-2}s^{-1}$ under 16 h photoperiod provided by cool white fluorescent light for two months.

4.6. Shoot regeneration and Rooting

Somatic embryos were carefully transferred to MS medium supplemented with 1 mg/l KN and 0.25 mg/l NAA for regeneration and followed by rooting on similar medium at the same time during shoot induction and at light intensity of 20 µmol $m^{-2}s^{-1}$ under 16 h photoperiod provided by cool white fluorescent light and data were recorded after 4 and 8 weeks for shoot regeneration and rooting respectively. The data for shoot regeneration and rooting was recorded with number of shoots per somatic embryo and number of roots per explants.

4.7. Acclimatization

Roots were washed with tap water to remove the agar and planted in plastic pots containing soil, compost and sand in a ratio of 2:1:1, respectively. Six plantlets per pot and a total of five pots

were used for each cultivar. The pots were covered with transparent plastic bag until the explants tend to adopt the external environment and kept in the green house. The plastic bag was removed after one week and the number of survived plants was recorded after six weeks.

4.8. Statistical analysis

Statistical analysis of quantitative data was analyzed using SPSS computer software of version 16 of one way ANOVA. A difference at probability level of $p>0.05$ 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. Callus induction

At the first round of callusing, all the cultivars (AAUFM-19), AAUFM-14, AAUFM-10 and AAUFM-20) formed white watery and soft calli in all 2,4-D concentrations (1 mg/l, 1.5 mg/l, 2 mg/l, 2.5 mg/l and 3 mg/l) after 4 weeks as shown in Fig.1. No callus was induced on growth regulators-free medium by any cultivar. In further callusing, the calli became a little bit harder and some of the cultivars started to induce somatic embryo as shown in Fig.2. High percentages of calli were obtained in all cultivars and in all 2, 4-D concentrations. All the explants (100 %) of cultivar AAUFM-19, AAUFM-14, AAUFM-10 andAAUFM-20 induced callus on medium containing 1mg/l, 1 mg/l and 1.5 mg/l , 2 mg/l and 2.5 mg/l, and 2.5 mg/l 2.4-D respectively (Table 1).

Table 1. Response of callus induction in different concentrations of 2, 4- D.

Cultivars	2,4-D (mg/l)	Callus induction (%)
AAUFM-19	0.0	0
	1.0	100
	1.5	98.3
	2.0	96.67
	2.5	98.3
	3.0	95
14(100002)	0.0	0
	1.0	100
	1.5	100
	2.0	98.3
	2.5	96.67
	3.0	98.3
AAUFM-10	0.0	0
	1.0	98.3
	1.5	98.3
	2.0	100
	2.5	100
	3.0	96.67
AAUFM-20	0.0	0
	1.0	98.3
	1.5	98.3
	2.0	98.3
	2.5	100
	3.0	98.3

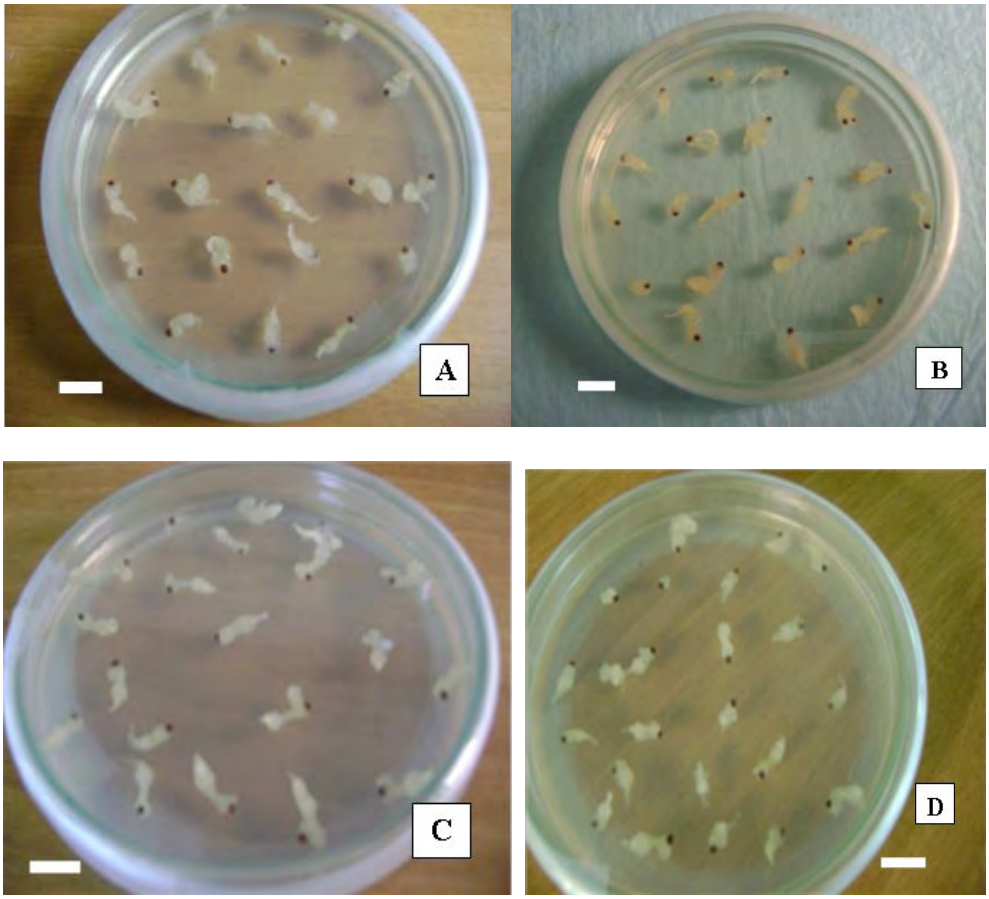

Figure 1. Callus induction after 4 weeks of culture in different concentrations of 2, 4-D for the four cultivars (A) AAUFM-10, (B) AAUFM-14, (C) AAUFM-20 and (D) AAUFM-19. Bars represent 2 cm.

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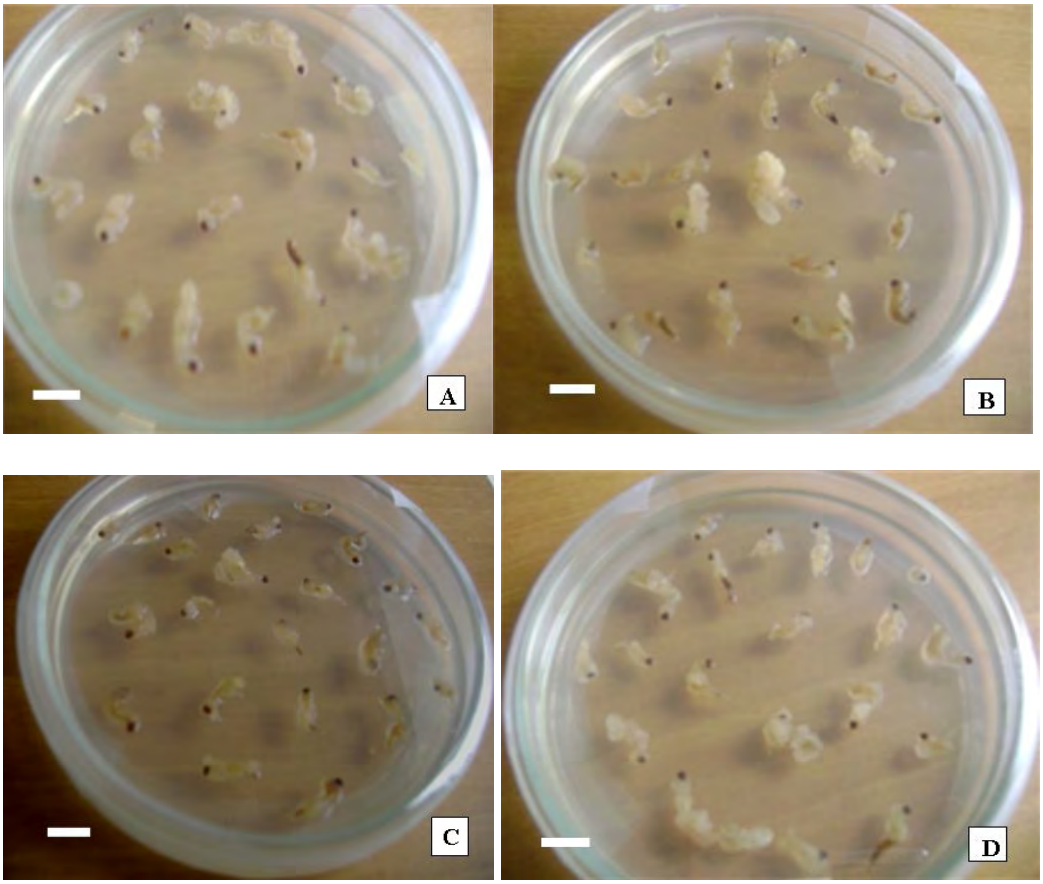

Figure 2.Callus induction from the four cultivars after eight weeks of culture in different concentrations of 2, 4-D.(A) AAUFM-10, (B) AAUFM-14, (C) AAUFM-20 and (D) AAUFM-19. Bars represent 2 cm.

5.2. Somatic embryogenesis

The eight-week-old calli of all the four cultivars were resulted in hard, compact, and gray and green mixed color somatic embryos after two months on medium containing 1mg/l 2,4-D (Fig. 3).

Table 2.Percentage of somatic embryo production from calli on MS medium containing different concentrations of 2, 4-D after two months of culture on MS-medium supplemented with 1 mg/l 2, 4-D

Cultivar	Concentration of 2,4-D in mg/l	No. of calli in culture	No.of calli forming SE	% of calli forming SE
14(100002)	1.0	60	27	45
	1.5	60	30	50
	2.0	59	31	52.5
	2.5	58	29	50
	3.0	59	27	45
AAUFM-10	1.0	59	29	49
	1.5	59	27	45
	2.0	60	35	58.3
	2.5	60	30	50
	3.0	58	30	51.7
AAUFM-20	1.0	59	31	52.5
	1.5	59	29	49
	2.0	59	27	45.7
	2.5	60	30	50
	3	59	27	45.7
AAUFM-19	1.0	60	30	50
	1.5	59	28	47.5
	2.0	58	33	56.9
	2.5	59	28	47.5
	3.0	57	32	56.1

A maximum percentage (52.5) and (58.3) of somatic embryo was obtained from calli induced on 2mg/l 2, 4-D in AAUFM-14 and AAUFM-10 respectively. Calli obtained on 1 mg/l 2, 4-D resulted in 52.5 % of somatic embryo in AAUFM-20 and calli obtained on 3 mg/l 2, 4-D produced 56.1 % of somatic embryos (Table 2).

Figure 3. Somatic embryogenesis after eight weeks of culture on 1 mg/l 2, 4-D. Bars represent 2 cm.

5.3. Shoot regeneration and rooting

All the cultivars have shown regeneration after four weeks of culturing on MS medium containing 1 mg/l KN and 0.25 mg/l NAA and rooting was also successful after eight weeks on the same medium. Multiple shoots were obtained in all cultivars.

Table 3.Shoot regeneration and rooting in the four cultivars of finger millet in calli resulted from different concentrations of 2, 4-D, on MS medium containing 1 mg/l KN in combination with 0.25mg/l NAA.

Cultivar	Concentration of 2,4-D (mg/l)	Mean No. of shoots/somatic embryo (±SE)	Mean No. of roots/explant (±SE)
14(100004)	1.0	9.70±0.80^{c}	5.89±1.01^{c}
	1.5	9.63±0.68^{c}	7.15±0.88^{c}
	2.0	10.67±0.75^{c}	6.15±1.18^{c}
	2.5	10.04±0.84^{c}	7.41±0.91^{c}
	3.0	10.30±0.78^{c}	6.93±1.19^{c}
AAUFM-10	1.0	13.37±0.74^{b}	11.85±1.18^{b}
	1.5	13.26±0.75^{b}	10.37±1.38^{bc}
	2.0	14.00±0.73^{ab}	11.89±1.26^{b}
	2.5	13.03±0.62^{b}	9.85±1.45^{bc}
	3.0	12.56±0.49^{b}	9.78±1.36^{bc}
AAUFM-20	1.0	13.48±0.59^{b}	13.00±1.68^{ab}
	1.5	13.19±0.59^{b}	11.11±1.48^{bc}
	2.0	12.59±0.62^{bc}	11.37±1.69^{bc}
	2.5	12.00±0.75^{bc}	9.85±1.47^{bc}
	3.0	10.93±0.55^{c}	10.19±1.58^{bc}
AAUFM-19	1.0	14.56±0.72^{ab}	16.74±1.86^{a}
	1.5	15.85±0.63^{a}	12.04±1.59^{b}
	2.0	14.41±0.52^{ab}	14.04±1.45^{ab}
	2.5	14.33±0.59^{ab}	16.59±1.71^{a}
	3.0	14.67±0.50^{ab}	11.15±1.81^{bc}

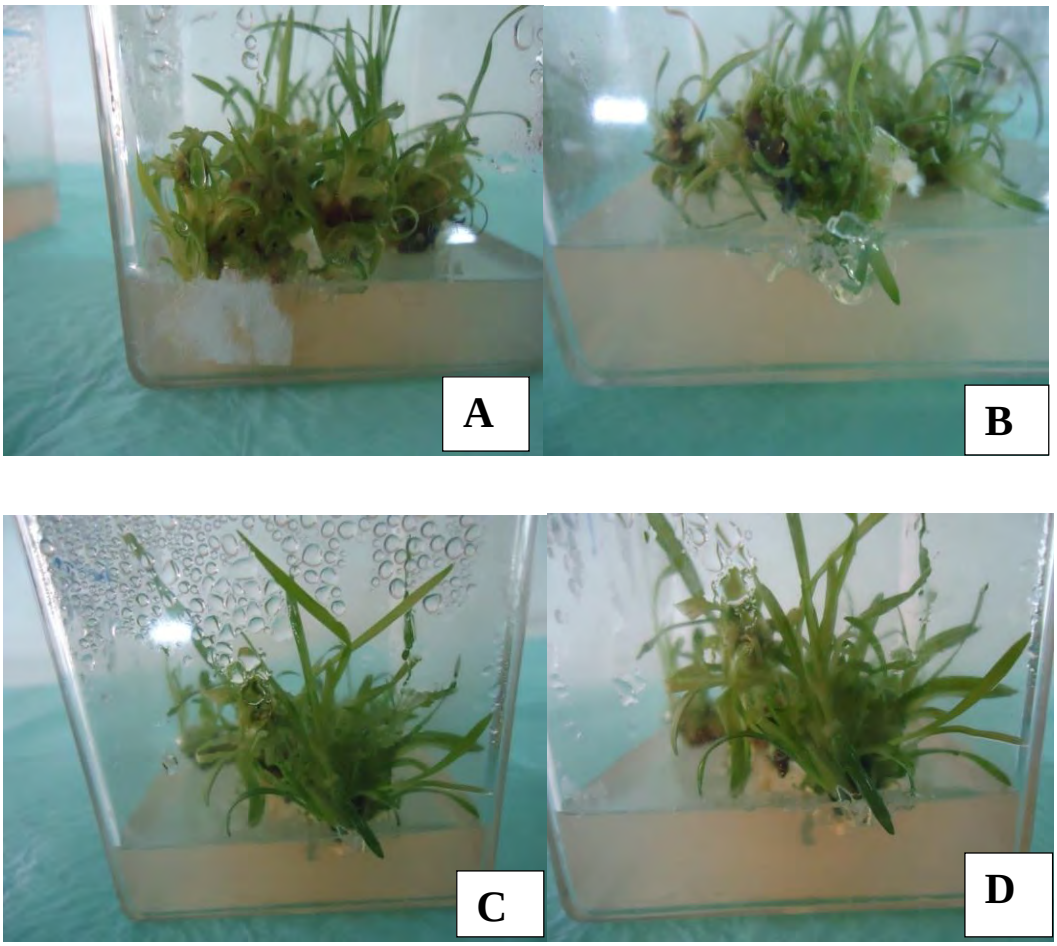

Figure 1. Multiple shoot regeneration from somatic embryo after four weeks of culture in MS-medium supplemented with 1mg/l KN in combination with 0.25mg/l NAA. (A) AAUFM-10, (B) AAUFM-14, (C) AAUFM-20 and (D) AAUFM-19

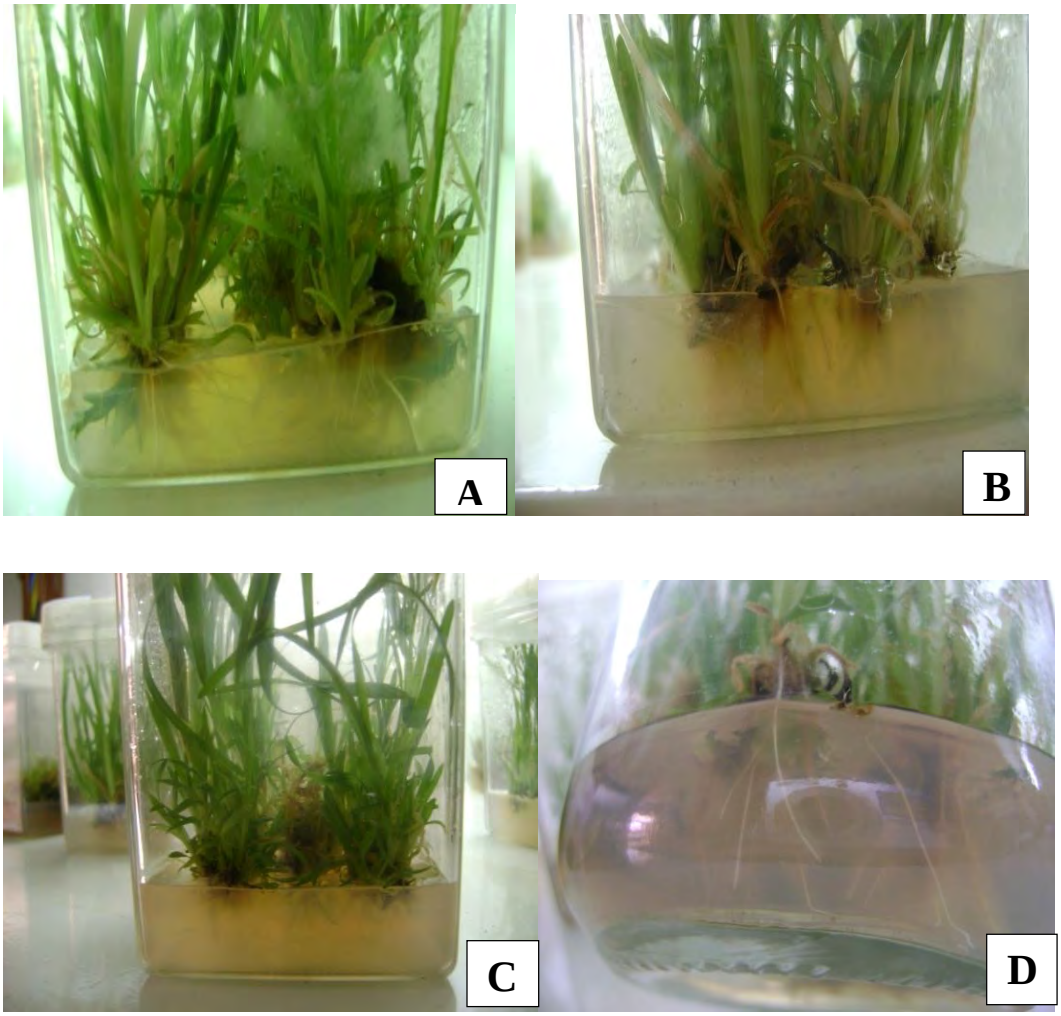

Figure 2. Formation of roots in four extended weeks after regeneration. (A) AAUFM-10, (B) AAUFM-14, (C) AAUFM-20 and (D) AAUFM-19.

5.4. Acclimatization

Plantlets of all four cultivars showed 100% survival after acclimatization (Fig. 7). During acclimatization, the plants produced additional shoots.

Figure 3. Acclimatized plants of the four cultivars of finger millet after 6 weeks

6. Discussion

6.1. Callus induction from seed culture

In this study, five different concentrations of 2, 4-D (1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) and growth regulators-free media were used for each cultivars (AAUFM-19), AAUFM-14, AAUFM-10 and AAUFM-20) for callus induction. All cultivars in all concentrations have shown nearly similar results with 95 to 100% callus induction. No callus was induced on growth regulators-free medium from any cultivar. Similar result was reported by Kebebew Assefa *et al.* (1998) on *Eragrostis tef* and Anjanuyulu *et al.*(2011) on finger millet. These authors pointed out that, 2, 4-D was found to be better than NAA for callus induction in these crops. According to Jabir and Siriwardana (1987) and George and Eapen (1990), the induction of callus in cereals and millets is commonly achieved by 2, 4-D. Faiz and Muhammad (2013) also reported similar result in different genotypes of rice with different concentrations of 2,4-D. However, they reported that the amount of calli increased with increasing the concentration of 2, 4-D, which was not the case in the present study.

6.2. Somatic embryogenesis

In this study, 1.0 mg/l 2, 4-D was used for somatic embryo induction from callus in all cultivars. Calli derived from different concentrations of 2, 4-D has shown difference in the efficiency of somatic embryo induction. Calli derived from 2.0 mg/l 2, 4-D exhibited 58.3% embryo formation in cultivar AAUFM-10 whereas 52. 5% somatic embryo formation was achieved from both AAUFM-14 and AAUFM-20 cultivars. On the other hand, 56.1% of somatic embryos were produced by calli derived from 1.0 mg/l 2, 4-D in cultivar AAUF-19. The above result indicates that there is a slight difference among cultivars within concentration of 2, 4-D in the efficiency of somatic embryogenesis. There was an influence of genotype, media, and explants on the amount of embryogenic callus formation. Jha *et al.* (2009) reported that callus derived from seed explants were initially loose and watery in nature and became embryogenic after three to four subcultures. On the contrary, callus induced from immature inflorescences were nodular, hard, and compact and had high regenerability. Shoot tip-derived callus was of inter-mediate quality and required two to three subcultures before becoming embryogenic and they reported that the maximum (80 %)of embryogenic callus was obtained from immature inflorescence-derived callus in pearl millet (*Pennisetum glaucum)* genotypes. In the present study, the highest percentage of somatic embryo formation was obtained at 1.0 and 2.0 mg/l 2, 4-D from seed-derived callus. In many plants, 2, 4-D has been widely regarded to be effective for somatic embryogenesis (Brown *et al.,* 1995). In addition to this, embryonic cells are easily formed on media supplemented with 2, 4-D for carrot explants (Jimenez and Bangerth,2001 as cited in Mousavizadeh *et al*.,2010) and also 2,4-D was able to produce high frequency of somatic embryos in *Sorghum bicolor* from leaf segments (Sudhakara and Sarada, 2006).

6.3. Shoot regeneration and rooting

In this step, somatic embryos of the four cultivars derived from different concentrations of 2, 4-D were transferred to 1 mg/l 2, 4-D for embryogenic callus formation and then transferred to a medium supplemented with combination of KN and NAA to evaluate their regeneration capacity. Different cultivars resulted in different mean number of shoots. The highest mean number of shoots (15.85) was obtained from calli derived from 1.5 mg/l 2, 4-D in AAUFM-19. The mean number of shoots, 10.67±0.75 and 14.00±0.73, shoots was obtained from calli derived from 2 mg/l 2, 4-D in cultivar AAUFM-14 and AAUFM-10 respectively. Calli derived from 1.0 mg/l 2, 4-D resulted in mean number of 13.48±0.59 plantlets in cultivar AAUFM-20.There was a difference in regenerative capacity between calli resulted from different concentrations of 2, 4-D. Somatic embryos of all cultivars obtained from calli that were induced on different concentrations of 2, 4-D induced plantlets in different frequency in MS-medium supplemented with 1.0 mg/l KN and 0.25 mg/l NAA. Rooting was proceeded in similar medium for further four weeks and different rooting efficiency was exhibited by different cultivars of finger millet. Mean number of roots, (7.41±0.91) was obtained from cultivarAAUFM-14 on medium containing 2.5 mg/l 2, 4-D whereas 11.89±1.26 roots were produced by cultivarAAUFM-10 on 2.0 mg/l 2, 4-D. Similarly, cultivars AAUFM-20 and AAUFM-19 produced 13.00±1.68 and 16.74±1.86 mean number of roots on medium containing 1.0 mg/l and 2.5 mg/l 2, 4-D, respectively. These results show that there was a significant difference among calli resulted from different concentration of 2, 4-D in the four cultivars of finger millet. Since the same treatment was used for all cultivars in the rooting medium, this difference could be due to genotype differences. The mean difference was significant at $p>0.05$among cultivars but there were no significant difference in regeneration capacity of calli derived from different concentrations of 2,

4-D in a specific cultivar. Genotype has often been considered to be an important factor in determining the response of *in vitro* regeneration (Vasil and Vasil,1983;Mahmuda *et al.,* 2003). KN in combination with NAA was playing an important role in giving multiple shoots and rooting efficiency in all cultivars. Similar result was reported by Anjaneyulu *et al.* (2011) in finger millet. They have reported that KN with low concentration of NAA resulted in high mean number of shoots and good rooting. Behzad *et al*. (2013) also observed that a positive effect of KN and NAA on root formation in *Matthiola incana.*

6.4. Acclimatization

All cultivars have shown 100% survival but there were some morphological differences among the cultivars in terms of number and appearance of shoots. In cultivar AAUFM-14 most of the plants only increased in height and a slight change in width of their shoots but there was no increase in number of shoots. Cultivars AAUFM-10 and AAUFM-20 exhibited increase in number and height of shoots.

7. Conclusion

Based on the above results, somatic embryogenesis and regeneration protocols have been developed through callus culture for four cultivars of finger millet, AAUFM-10, AAUFM-14, AAUFM-20 and AAUFM-19.

- 2, 4-D was appeared to be effective for callus induction at different concentrations for all cultivars. It was used in different concentrations (1-3 mg/l) for all cultivars of finger millet and resulted in 95-100% of callus induction in each cultivar.
- For somatic embryogenesis, a medium supplemented with 1 mg/l 2, 4-D was found to be the best concentration in the four cultivars of finger millet.
- Best shoot regeneration and rooting was obtained on MS-medium supplemented with1 mg/l KN and 0.25 mg/l NAA in all cultivars of finger millet.
- There was a significant difference between cultivars but there was no significant difference in calli derived from different concentrations of 2, 4-D in a specific cultivar.
- Acclimatization was 100 % successful and all the plants were healthy in all cultivars.

8. Recommendations

Based on the results of this study, the following points have been recommended,

- PEG will be important to test in different cultivars of finger millet for in vitro screening of drought tolerance.
- In this study somatic embryogenesis protocol is developed for four cultivars of finger millet and this is useful to do different biological improvements in these cultivars.
- Although finger millet is economically important, especially in the developing world, little genetic improvement has been done so far specifically using wide- or cross-hybridization among closely related species. Therefore it is important to do modern genetic improvements through transformation.
- One of the most common barriers of finger millet production is blast disease. So it is very important to develop blast resistant variety of finger millet. To do such improvement and to make other genetic transformations to the crop, this somatic embryogenesis protocol is very important.

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10. Appendices

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

Macronutrients	g/l	Micronutrients	mg/500ml	Vitamins	mg/500ml
KNO3	38	FeSO4.7H2O	2780	Myoinositol	1000
NH4NO3	33	Na2EDTA.2H2O	3730	Glycine	200
CaCl2.2H2O	8.8	ZnSO4.7H2O	430	Nicotinic acid (NaOH)	50
MgSO4	7.4	H3BO3	630	Pyridoxine (B6)	50
KH2PO4	3.4	MnSO4. 4H2O	2230	Thiamin (B1)	10
		CuSO4. 5H2O	2.5		
		KI	83		
		Na2MoO4.2H2O	25		
		CoCl2. 6H2O	2.5		

Appendix 2. ANOVA result for number of shoots/somatic embryo and number of roots/explants

				Sum of Squares	df	Mean Square	F	Sig.
Shoots/SE	Between Groups	(Combined)		1760.776	19	92.672	7.633	.000
		Linear Term	Contrast	1015.715	1	1015.715	83.659	.000
			Deviation	745.061	18	41.392	3.409	.000
	Within Groups			6313.407	520	12.141		
	Total			8074.183	539			
Roots/explant	Between Groups	(Combined)		4784.222	19	251.801	4.562	.000
		Linear Term	Contrast	2880.806	1	2880.806	52.196	.000
			Deviation	1903.417	18	105.745	1.916	.013
	Within Groups			28699.778	520	55.192		
	Total			33484.000	539			