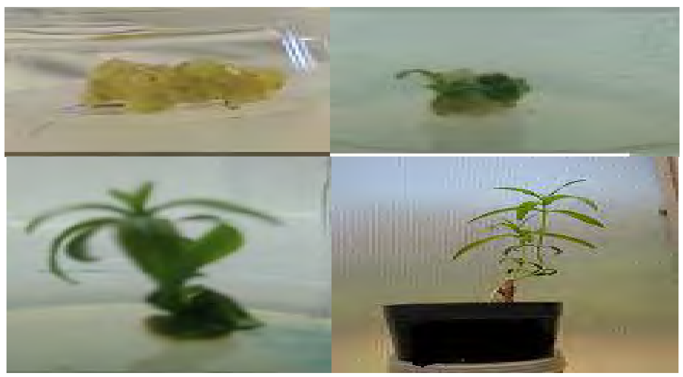
# Addis Ababa University
# School of Graduate Studies

# Plant regeneration from anther culture of niger (*Guizotia abyssinica* (L.f) Cass.)



**A thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)**

**By**

**Misteru Tesfaye Woldeyohannes**

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## ABBRIVATIONS

$B_5$ -- Gamborg medium

BAP -- 6-Benzylaminopurine

2, 4-D -- 2, 4-Dichlorophenoxy acetic acid

EC -- Embryogenic callus

HARC -- Holetta Agricultural Research Center

IBA -- Indole butyric acid

KN -- Kinetin

LS --Linsmaier and Skoog medium

MS -- Murashige and Skoog medium

$N_6$ -- Chu medium

NAA -- Naphtalene acetic acid

NEC -- Non- embryogenic calli

PGR -- Plant growth regulator

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## ABSTRACT

Niger (*Guizotia abyssinica* (L.f) Cass) is an important oilseed crop in Ethiopia. The out-crossing and self-incompatibility nature of the crop make breeding works difficult. The application of anther culture in niger improvement is very important in developing haploids or double haploids. Double haploids enable to increase the selection efficiency of desirable traits in recurrent selection scheme or used in producing synthetic cultivars. Identification of the best responsive genotypes and appropriate media for callus induction are prerequisite to regenerate plants using anther culture technique that supports the breeding program. Anthers of four niger varieties namely Shambu, Kuyu, Esete and Fogera were cultured into four different basal media (MS, $B_5$, $N_6$ and LS). All basal media were supplemented with 2 mg/l 2, 4-D + 0.3mg/l KN or 2 mg/l NAA + 0.3 mg/l KN. Identification of the appropriate stage of harvesting capitula and optimization of the time duration of cold-pretreatment was undertaken using anthers of Shambu. Callus induction experiment was also conducted on MS, $B_5$ and NN media to identify the types of calli induced. Various experiments were carried out to optimize callus induction, shoot regeneration and rooting of plantlets using different combinations and concentrations of PGRs. Data such as callus induction percentage, types of induced calli and regeneration percentage of shoots and roots were collected and analyzed. Capitula (buds) fully or slightly covered by sepal having whitish- green or greenish-yellow anthers were the optimum stages of harvesting capitula and 24 hours time duration was recommended for cold pretreatment at $4^0$C. ANOVA showed that genotypes and media significantly affect callus induction percentage and regeneration. The effect of the two factors, however, is independent. The average callus induction percentage was highest in Shambu (29.4%) followed by Fogera (21.4%) in the same treatment. It was possible to distinguish embryogenic calli (EC) from non-embryogenic calli (NEC) by visual observation. NEC were identified by their friable morphology and amorphous texture, which were dominantly developed in MS media. Embryogenic calli were mainly produced in NN and B5 media, which were distinguished by their variable texture and colour between the two media. Among ten and nine different PGRs combinations and concentrations, 2 mg/l 2,4-D + 0.3 mg/l KN and 2 mg/l KN + 1mg/l IAA were found to be superior PGRs combinations for callus induction and for shoot regeneration respectively. It was also found that 0.5mg/l IBA was the better PGR for rooting. Regenerants of Shambu seem to be having better survival rate than the variety Fogera either in the growth room or glass house.



**Key Words / phrases: *Guizotia abyssinica* (L.f) Cass., anther culture, embryogenic callus, non-embryogenic callus, basal media, plant growth regulators.**

# 1. INTRODUCTION

Niger (*Guizotia abyssinica* (L.f ) Cass.) is an oil seed crop, indigenous to Ethiopia where the crop is found in greatest diversity. Taxonomically niger belongs to the family Compositae and genus Guizotia (Baagoe, 1974). Niger ('Noug' in Amharic) may have originated from the wild species *Guizotia scabra* subsp. *schemperi* ('Mech' in Amharic), a common weed in Ethiopia through disruptive selection by Ethiopian farmers several years ago (Abebe, 1992). Niger is mainly cultivated in Ethiopia and India. It is also cultivated in small scale in other African countries such as Sudan, Uganda, Zaire, Tanzania, Zimbabwe and Malawi (Weiss, 1983).

In Ethiopia, niger is mainly grown from 1600 masl to 2500 masl, where the average temperature ranges from $15^0$C to $23^0$C and annual rainfall ranges from 500 mm to 1000 mm (Getinet and Sherma, 1996). Gojjam, Gondor, Shoa and Wellega are the major niger producer regions and Wello, Harerge, Arsi and Bale regions also cultivate this crop (Getinet and Nigussie, 1992).

In Ethiopia niger can be grown as a sole crop but often intercropped or rotated with other crops mainly with 'teff' (*Eragrostis tef*) and maize (Hiruy Belayneh, 1987) usually without the application of fertilizer or herbicides. Too much fertilizer promotes vegetative growth rather than increasing seed yield (Hiruy and Nigussie, 1985). Niger can grow on a wide variety of soils, buts appears to thrive best on clayey loam or sandy clays (Weiss, 1983) within a pH ranges 5.2-7.3 ( Getinet and Sherma, 1996).

Niger is characterized by indeterminate growth habit (Weiss, 1983; Riley and Hiruy Belayneh, 1989). Correct time of harvesting of niger needs consideration because ripe buds and young flower may be present on the same plant at harvest which result in uneven ripening that cause loss from seed shattering (Hiruy Belayneh, 1987).

Niger is a major source of edible oil in Ethiopia. It also contributes in edible oil production in some African and Asian countries, mainly India (Weiss, 1983). Niger oil is not only used for edible purposes but also used for industrial purposes in preparation of soaps, paints, illuminants and lubricants and for cleaning machinery (Baagoe, 1974; Riley and Hiruy Belayneh, 1989). The press-cake left over after oil extraction is an excellent poultry and livestock feed as it contain 33-37% protein and is rich in inorganic constituents and crude fibers (Seegler, 1983; Jeff *et al*, 2002)

Despite its considerable importance, niger has low seed yield due to several factors. Seed shattering, lodging, uneven maturity of heads and self-incompatibility are the major factors for its low productivity. Pests and diseases are also other factors for niger seed productivity even if the crop possesses apparently considerable level of tolerance for such factors, which needs to be well investigated (Weiss, 1983).

Niger is highly out- crossing species with self- incompatibility mechanisms (Sujatha, 1993) and insects, particularly bees, are the major agents of pollination. Because of its self -incompatibility nature, breeding procedure used in the improvement of cross-pollinating crops are the methods of choice for niger breeding (Doggett, 1987). In Ethiopia, recurrent selection methods namely mass and half-sib recurrent selection methods have been employed for several years for the development of an early to medium maturing, short plant type variety (Getinet and Sherma, 1996).

The self-incompatibility nature of niger causes serious difficulty for inbred line development which is essential for breeding purposes. Although Chavan (1961) reported the possibility of maintaining the purity of niger breeding materials by forced inbreeding or by sib-mating using, this method gave very small mount of selfed seed and it was found to be laborious and time taking. Studying anther culture of niger is beneficial for *in vitro* haploid plant production, which in turn used as the better alternative method for inbred line development of niger in short period of time. Such lines can also be used in niger breeding program as a parental or test cross lines or used in development of synthetic cultivar of niger population with desirable trait.  Double haploids from anther culture of niger can also be used to improve the selection efficiency of quantitative character by incorporating them in recurrent selection cycle. Despite its importance, little has been done in anther culture of niger. In India, dwarfs and large headed self-compatible double haploid niger genotypes were obtained from anther culture (Saverish *et. al,* 1993b, 1994a). However, so far anther or microspore culture is not exercised on Ethiopian genotypes.

Success in anther culture method depends on several factors such as growth conditions of the plant, genotypes of the plant, basal media and plant growth regulators used. Each factor becomes critical in callus induction and plant regeneration. Anther culture studies by Saverish *et al* (1993b) indicated the effect of basal media and plant growth regulator in callus induction and shoot regeneration in anther culture on Indian niger genotypes. The effect of such factors is genotype dependent (Swapan, 2005) and thus it is important to optimize the above and other factors that affect anther culture of Ethiopian niger genotypes.

# 2. LITERATURE REVIEW

## 2.1 Description of the crop

### 2.1.1 Morphological description

Niger is a stout, erect, moderately branched and annual dicotyledonous herb with attractive flowers, whose seed produces edible oil. Germination is epigeal and seedlings have pale green to brownish hypocotyls and cotyledons (Seegeler, 1983).

The root system is usually well developed, with a central taproot and branching laterals, but since the plant can grow on poor or stony soils, roots seldom develop to their maximum. The stem of niger is round, smooth to slightly rough and bearing small and soft hairs. The color of the stem varies from dark purple to light green. An average of 1.4 m plant height of niger with five to twelve primary branches have been reported by Getinet and Sherma (1996). The leaves are opposite sometimes alternate on the upper stem, sessile, lanceolate, 10-20 cm in length and 3-5 cm in width, softly hairy on both surfaces. The leaf margin morphology varies from pointed to smooth and leaf colour varies from light green to dark green.

The flower head of niger sometimes called capitulum is composed of disc and ray florets. Each disc floret has one stigma and five fused lobe stamens and is thus, hermaphroditic. The ray florets (usually eight in number) are formed on the outer circle of the head and pistillate. They bear a single petal-like structure, which decorates the entire flower head with deep yellow coloration (Riley and Hiruy, 1989). The hermaphroditic disk florets, usually 40-60 per capitulum, are arranged in three whorls, those at the edge opening first, followed progressively by the next in line to the center of the head like sunflower. Flower anthesis begins early in the morning at about 5:30 am and dehiscence of pollen begins 2 hours later and continues up to 10:00 am under condition at Holetta, Ethiopia (Adefris and Nigussie, 2002). The style emerges covered with pollen but dehiscence of pollen takes place earlier (protandry) and there is a delay in the exposure of the receptive part of stigma. This condition could partly be the reason for cross-pollination and self-incompatibility behavior of niger (Adefris and Nigussie, 2002). Insects, particularly bees, are the major agents of pollination (Ramachandran and Menon, 1979)

### 2.1.2 Chemical properties

The oil content of niger seed varies widely, between 25 and 45% for unimproved types and 50-60% in selected strains (Weiss, 1983). The Ethiopian niger seeds contain fatty acid composition of 75-80% linoleic acid, 7-8% palmitic and stearic acids, and 5-8% oleic acid (Getinet and Adefris, 1995). The Indian types contain 25% oleic and 55% linoleic acid (Nasirullah *et al*., 1982 cited in Getinet and Sherma, 1996). Protein content of niger can vary from 12 to 50% (Weiss, 1983).

## 2.2 Distribution and origin of niger

According to Baagoe (1974) the genus *Guizotia* in which the cultivated species *Guizotia abyssinica (L.f*) Cass and others related species are found, is native to tropical Africa. *Guizotia villosa* is concentrated in the northern and southwestern highlands of Ethiopia (Mulatu Geleta, 2007). *Guizotia zavattarii* is endemic around Mount Mega in southern Ethiopia and the Huri hills in Northern Kenya. *Guizotia arborescens* is endemic to the southwest of Ethiopia and Imantong mountain areas on the border between Sudan and Uganda. *Guizotia scabra* subsp. *scabra* is distributed from Ethiopia to Zimbabwe in the south and the Nigerian highlands in the west, dissected by the Sudanese desert and Congo rainforest. *Guizotia reptans* is endemic to Mount Kenya, the Aaberdares and Mount Eligon in East Africa and is the only taxon, which is not reported from Ethiopia (Kifle Dagne, 1994b).

Cultivated niger *Guizotia abyssinica* (L.f) Cass may have originated through disruptive selection from *Guizotia scabra* subsp.*schemperi,* a common weed in Ethiopia (Doggett, 1987). It is believed to have been taken to India by Ethiopian immigrants, probably in the third millennium BC along with other crops such as finger millet (Doggett, 1987). Niger is also found in some areas in Sudan, Uganda, Democratic Republic of Congo, Tanzania, Malawi and Zimbabwe, and the West Indies, Nepal, Bangladesh, Bhutan and India (Weiss, 1983).

### 2.3 Ecology, production status and agronomy of niger

Niger is basically a cool season or temperate region crop. The major niger producing areas in Ethiopia are characterized by moderate temperature ranging between $15^0$C and $23^0$C during the growing seasons (Getinet and Sherma, 1996). In Ethiopia, niger is grown mainly in mid-altitude and highland areas (1600-2500m) bisected by $10^0$N latitude, although it is sown in small patches in most regions of that country. In India it grows well on the plains and up to 2000 m in more hilly districts. There is thus a basic difference in environment between Ethiopian and Indian types, which are sufficiently differentiated in their reaction to specific climatic conditions, day length, temperature, rate of growth etc. confirmed by green house trials (Mesfin *et al.,* 1978). According to Getinet and Adefris (1995), niger requires moderate temperature that ranges 18-$23^0$C and above $30^0$C.The rate of growth and flowering are adversely affected and maturity accelerated. Niger requires adequate rainfall over the main growing period to produce a commercially acceptable yield. It is not primarily a dryland crop although it is often grown in low rainfall areas, but this is usually due to the necessity to use more fertile land for food crops. Niger is adapted to areas where rainfall does not exceed 1000mm per year (Getinet and Sherma, 1996). Niger can be found growing on a wide range of soil types, but appears to thrive best on clayey loams or sandy clays (Weiss, 1983). In most highlands of Ethiopia, niger is grown on the soil having dark or dark brown color. Niger does not prefer poor soil but it is usually sown on stony or lateritic soils as in India (Weiss, 1983). It grows well at pH values between 5.2 and 7.3.

Niger is the most important oil crop contributing 50-60 % of the oilseed production in Ethiopia (Riley and Hiruy, 1989) and 2% in India (Quinn and Mayer, 2002). According to the later authors, India is reported to have total niger annual production of 200,000 tonnes, with the vast majority being exported to Europe, Japan and the US for the birdfeed market. Although it is difficult to state the accurate production status of niger in

Ethiopia, Gojjam, Showa, Gondor and Welega are the major niger production areas accounting generally for about 90% of the country's total production (Hiruy and Nigussie, 1985). The remaining 10 % is produced in Wello, Hararghe, Arsi and Bale.

Land preparation for small grains such as sorghum, wheat and millet is suitable for niger but in areas where niger is grown as in intercrop, lands are cultivated to suit the most important component, with the result that niger frequently suffer from unsuitable land preparation. In Ethiopia, the growing season of niger varies with the type of landrace found in the area (Hiruy and Nigussue, 1987). Farmers in the central highland, where the 'abat' niger is mainly grown, plant niger in mid-May to early June and harvest in December, the 'Bungne' niger of low land type is planted in July and harvested in October and the growing season of 'mesno' niger, short season type of highland, is from September to February. Niger is a small-seeded crop and the seed rates vary from 5-10 kg/ha in Ethiopia and from 5 to 8 kg/ha in India (Getinet and Sherma, 1996). The most usual method of sowing is by broadcasting but it can also be sown in rows. In Ethiopia niger is mainly sown as a sole crop, usually in rotation with ' tef ' and maize (Getinet and Sherma, 1996). According to fertilizer requirement studies of niger by Hiruy and Nigussie (1987), niger is less responsive to nitrogen and phosphorous fertilizer. It is, however, recommended 23 kg N/ha and 23 kg $P_2O_5$ /ha for initial growth of the crop. In Ethiopia, farmers often report that niger is a good precursor for cereals and the crop following niger have less weed infestation (Hiru Belayneh, 1987). Niger normally matures in 120 -180 days after emergence in Ethiopia and Indian cultivars mature in 75-120 days (Weiss, 2000). Results of harvesting study of niger in Ethiopia showed that niger should be harvested when the buds turn from yellow to brown or at bud moisture content of 40 -50% (Hiruy and Nigussie, 1987). Harvesting of niger at the appropriate time is very important in reducing loss of yield through shattering.

### 2.4 Cytogenetics of *Guizotia abysssinica* (L.f) Cass.

Niger (*Guizotia abyssinica* (L.f) Cass.) is a diploid species with 2n = 30 chromosome (Kifle Dagne and Haneen, 1992 and Kifle Dagne, 1995). *G.abyssinica* showed karyotype heterogeneity in terms of number of satellite chromosomes and median and submedian chromosomes (Kifle Dagne and Heneen, 1992). The number of median chromosomes ranged from 18 to 26 and that of submedian chromosomes from 2 to 10. The karyotype of *G.abyssinica* and *G.scabra* subsp.*schimperi* are similar although the latter has relatively smaller chromosomes (Kifle Dagne and Haneen, 1992; Kifle Dagne, 1995). The differences in size of their chromosomes explain the variation in their genome size as reported by Hiremath and Muthy (1992).

### 2.5 Mode of reproduction of niger.

Niger is a completely outcrossing species with a self-incompatibility mechanism (Murthy *et al*., 1993). Detail evidence regarding self–incompatibility for *Guizotia abyssinica* was reported by Sileshi Nemomissa *et al* (1999). According to these authors, self-incompatibility in niger is of the sporophytic type that causes inhibition of pollen germination or twisting of pollen tube over the surface of papillae. Self-compatible niger genotypes were also reported in low frequencies within the Ethiopian gene pool, which can be as high as 5% in some populations (Getinet and Sherma, 1996; Sileshi Nemomissa, 1999). Insects, particularly bees, pollinate niger (Adefris and Nigussie, 2002).

### 2.6 Economic importance

Niger seed is used as a human food. The seed is roasted and crushed with a pestle in a mortar and then mixed with crushed pulse seed to prepare ‘wot’ in Ethiopia (Seegler, 1983). In Ethiopia, niger is mainly cultivated for its edible oil. The traditional method for extraction of oil from niger in Ethiopia is through a combination of roasting, grinding and mixing with hot water followed by shaking in a container made of clay. The oil may be used in cooking, anointing the body, adulterating more valuable oils, painting and cleaning of machinery (Seegler, 1983). Niger is used to generate income for the farmers either in local market or by exporting abroad. The oil from niger also substitutes for sesame oil for pharmaceutical purposes and can be used for soap making (Getinet and Sherma, 1996). Niger has become as a specialty grain opportunity that exported for bird food market especially in Midwestern US (Quinn and Mayer, 2002). The meal remaining after the oil extraction contains about 24% protein and 24% crude fiber (Seegler, 1983). It is a good feed for livestock.

## 2.7 Production and research constraints

### 2.7.1 Genetic factors

The national average productivity of niger is generally very low. Current reports state that the national productivity of niger is not more than 4.2 Q/ha ( Bulcha, *et al*., 2007). One of the most important factors for niger low productivity is genetic. Most of the national production of niger is mainly based on local land race populations, which have low yielding capacity. According to Getinet and Sherma (1996), three landraces of niger are known in Ethiopia. These are 'abat' 'bungne' and 'mesno', which are late maturing, early maturing and late maturing respectively. 'Abat' is the common landrace with higher yield than the other two landraces and with higher oil content than 'bungne' (Getinet and Sherma, 1996).

Lodging, shattering, indeterminate growth habit and self-incompatibility have been reported as major factors that contribute to low yield in *G. abyssinica*. The indeterminate growth habit of niger favors seed shattering since those buds ripen first begin to shed their seed before the last heads are mature. The self-incompatibility of niger hinders the production of selfed seed and makes the breeding work difficult, which in turn hamper seed yield improvement of niger.

### 2.7.2 Parasitic weeds, insect pests and diseases of *G. abyssinica*

Parasitic weed namely dodder (*Cuscuta campestris*) is a major threat in niger production both in Ethiopia and India (Fessehaie, 1992; Getinet and Sherma, 1989). Even if insect pests and diseases of niger are fewer as compared to other oil crops, Getinet and Sherma (1996) reported a large number of insect pests and diseases of niger. Niger fly (*Dioyna sororcula* and *Eutretosoma* spp.), and black pollen beetles (*Meligethes* spp.) are the most important insect pests of niger while niger blight (*Alternaria* sp.) and bacterial leaf spot (*Xanthomonas* sp.) are the most serous diseases of niger (Getinet and Sherma, 1996).

## 2. 8 Niger Breeding

### 2.8.1 Breeding objectives

Niger to be competitive with other oil crops such as linseed and Ethiopian mustard, its yield must be significantly improved. To achieve this objective, less branching, shorter cultivars with uniform flowering must be developed. Although the number of branches is positively correlated with yield, excessive branching is usually related to increasing in vegetative growth that promotes lodging and in fact decreases rather than increasing seed yield (Getinet and Sherma, 1996). According to Hiruy Belayneh (1987), shorter plants will have less vegetative growth or number of leaves that contribute for a better harvest index. Therefore, the first main objective of niger breeding is to increase seed yield by developing less branching, shorter plants and uniform maturing cultivars. The second most important breeding objective of niger improvement is increasing the seed oil content. There exists great genetic diversity for oil content in Ethiopian and Indian germplasm collections which could be used, in breeding program, to significantly increase oil content (Getinet and Adefris, 1995).

### 2.8.2 Breeding methods

The breeding method of crops depends on its pollination behavior and the structure of its reproductive system. In crops in which cross-pollination is predominant, there is great phenotypic variability or it is difficult to find uniform cultivars or identical genotypes due to extensive heterozygosity. Even if the genetic variability for qualitatively inherited characters may be drastically reduced by rigid selection, genetic variability in quantitatively inherited character continues to present, due to inability of the breeder to select accurately for individual gene effects and to influence the genotype by environment interaction. Thus, breeding procedures used in highly cross pollinated crops like niger are based largely on population improvement by giving more emphasis to quantitatively inherited characters (Allard, 1960). The resulting varieties are open-pollinated varieties.

Recurrent selection is one of the breeding methods usually applied in cross-pollinated crops for the release of open-pollinated varieties (Allard, 1960). Mass and half-sib recurrent selections are powerful tools used in niger improvement (Getinet and Adefris, 1992). The principle of mass and half-sib of recurrent selection is the same just to increase the frequency of desired alleles for particular quantitatively inherited characters by repeated cycles of selection. In general, mass and half-sib recurrent selection methods have been successfully employed for the development of early to medium maturing, big head size and shorter plant type varieties of niger (Getinet and Sherma, 1996). So far four varieties namely Fogera, Esete and Shambu have been released, in Ethiopia.

## 2.9 Biotechnological studies in niger

### 2.9.1 Molecular marker

Molecular markers are one of the biotechnological approaches, which might be used in niger improvement. However, little has been done in these areas. Mulatu (2007) has identified twelve AFLP and four RAPD taxon specific DNA markers. These markers have a potential use in *Guizotia abyssinica* breeding since they might be linked to desirable traits that are worth transferring to *G. abyssinica.* Such taxon specific DNA markers in *Guizotia* might also be used in species identification and characterization of niger in gene bank. Isozyme assessment study of the relationships between *G. abyssinica* and its close wild relatives has been conducted by Legesse (2006). According to his study, the genetic variation within or among taxa indicated the presence of a close relationship between *G. abyssinica* and its close wild relatives. The result of the genetic divergence between these related groups of taxa was in agreement with the morphological and cytological studies.

### 2.9.2 Tissue culture in niger

Simmonds and Keller (1986) developed plant regeneration protocol of niger from leaf tissue. Optimal cultural conditions for regeneration of plantlets from cotyledon and hypocotyl of niger were demonstrated by Saverish *et al* (1997) and Tesfaye Dissasa (2007). According to these studies plant regeneration was dependent on genotypes and media composition used. The protocol for *in vitro* plant regeneration for a large scale propagation of male sterile niger was reported by Sujatha (1997). This protocol enables to maintain the male sterile plants of niger through direct adventitious shoot regeneration using leaf tissues from mature plants. Although efforts to develop dihaploids of niger from ovule culture were unsuccessful, self-compatible lines, dwarfs and single headed double haploid plants were obtained from anther culture of niger in India (Getinet and Sherma, 1996).

## 2.10 Androgenesis

Androgenesis is a system of achieving the regeneration of complete plants through male gametes, anthers or microspore culture *in vitro.* Male reproductive processes take place in the stamens (anthers) in flowering plants. The diploid cells undergo meiosis and produce haploid male spores or microspores. Microspores divide mitotically and differentiate into multicellular male gametophyte or pollen grains. The principle of androgenesis is to arrest the development of the microspore (male gametophyte) and to force them towards somatic (sporophytic) pathway (Swapan, 2005).

According to the early cell division study of anther culture by Reyonlds (1990), for species cultured during the uninucleate stage, the microspore either undergo a normal mitosis and form a vegetative and a generative nucleus or divides to form two "similar-looking" nuclei. In those cases where vegetative and generative nuclei are formed in culture, or where binucleate microspores are placed into culture, it is usually the vegetative nucleus that participates in androgenesis (Chen, 1986). The only species in which the generative nucleus has been found to be actively involved in androgenesis is black henbane (*Hyoscyamus niger* L.) (Datta, 1998). This study also stated that the two similar looking nuclei or one of them might undergo further divisions. In some cases they may fuse and produce homozygous diploid plants or callus. Since diploid callus may also arise from somatic tissue associated with anther, the haploid of anther culture should have been verified for their haploidy either by chromosome counting or using flow cytometry technique (Chen, 1986).

### 2.10.1 Anther or microspore culture

Anthers contain pollen and when the anthers are cultured intact, the procedure is called anther culture. Microspore culture involves isolating the microspores from anthers before culturing and is sometimes referred to as pollen culture. *In vitro* androgenesis leads to the formation of haploids either by direct embryogenesis or indirectly through callus phase (Sandra, 1996). Direct androgenesis mimics zygotic embryogenesis with the exception that a true suspensor is not involved in androgenic embryo (Datta, 1988). At the globular stage of development, most of the embryos are released from the pollen cell wall (exine). They continue to develop, and after 4-8 weeks, the cotyledons unfold and plantlets emerge from anthers. Such kind of embryogenesis is common in Solanaceae (e.g tobacco) and Cruciferae (e.g. mustard) families (Sandra, 1996). During indirect androgenesis, the early cell division pattern is similar to that found in the zygotic embryogenic and direct androgenic pathways (Datta, 1988). After the globular stage, irregular and asynchronous divisions occur and callus is formed. This callus must then undergo organogenesis to regenerate haploid plantlet under the influence of growth hormone and the culture media. Indirect androgenesis is common in cereals and callus-derived haploids usually exhibit generic variation and polysomy (Swapan, 2005).

Although it is clear that direct culture of microspore will ultimately provide the most efficient procedure for the production of haploids, anther culture remains a valuable starting point for studies of haploids since it is relatively simple and fast method and requires only minimal facilities (Jahne and Lorz, 1995). Anther culture also constitutes an important experimental tool for investigation of fundamental questions relating to the growth of haploid embryoids and the regeneration of plants. Therefore, studies on the *in vitro* culture conditions for anther culture of niger was attempted in the present study.

### 2.10.2 Factors affecting anther culture

*Condition of donor plants*

The age and physiological condition of donor plants often affect the outcome of anther culture experiment (Wan and Widholm, 1989). In most cases, the best responsive stage of the plant is the first set of flowers and anthers should be cultured from buds collected as early as possible during the course of flower (Sandra, 1996). Various environmental factors such as light intensity, photoperiod and temperature may also affect haploid plant production through anther culture. The optimum conditions of such factors have been investigated for some species such as rice, wheat and barely (Swapan, 2005). In general, controlled light, temperature and humidity, which enable plants to maintain a healthy growth with disease and pest free status, give a high degree of success of anther culture response with reproducible results (Wan and Widholm, 1989).

*Genotype*

A genotype grown in a particular environment plays an important role in androgenic response. The degree of response for anther culture varies either among species or within a species. For example, some maize (*Zea mays* L.) cultivars are completely unresponsive in anther culture, whereas a few haploids can be obtained from others cultivars (Wan and Widholm, 1993). Several studies indicate that anther culture response of a genotype is influenced by gene combinations (Swapan, 2005). Because of this genotypic effect, it is important to include as much genetic diversity as possible when developing protocols for producing haploid plants through anther culture.

*Microspore stage*

The most critical factor affecting haploid plant production from anther culture is the stage of microspore development. For many species, anthers are only responsive during the uninucleate stage of microspore development (Datta and Wenzel, 1987). In contrast, optimum response is obtained in tobacco and maize during late uninuceate to early binucleate microspoes (Gaillard *et al*., 1991; Sandra, 1998).

In developing a protocol for anther culture, external staging system of flower heads based on measurements of physical characteristics has been found to be essential (Sandra, 1996). Such measurements may include calyx and corolla length, anther colour and anther shape and size. The physical description of buds and anthers are then examined and used to determine the bud stage corresponding to the optimal stage of microspore development for anther culture (Jahne and Lorz, 1995).

*Pretreatment or preincubation*

The induction of microspores to sporophytic instead of gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before anther culture (Swapan, 2005). In many species, cold or hot pretreatment before excision of anthers has been found to be essential to improve the androgenic response (Sandra, 1996). Such kinds of pretreatments are species and even genotype dependent. Yield of tobacco haploids, for example, are often increased by storing excised buds at 7-8$^0$ C for 12 days prior to anther excision and culture (Senderland and Roberts, 1979). According to Sandra (1996), the temperature from 4-10 $^0$ C and duration from 3 days to 3 weeks have been usually utilized for most crop species. High temperature pre-incubations have been effective in some species. For example, haploid plant production was increased in rape (*Brassica campestris* L.) by culturing the anther at $35^0$ C for 1-3 days prior to culture at $25^0$ C (Keller and Armstrong, 1979).

*Culture media*

The nutrient medium not only provides nutrition to the microspores but also directs the pathway of embryo development. For most species, the most commonly used media for anther culture are MS ( Murshaige and Skoog, 1962) and $N_6$ (Chu, 1978) or variations on these media. In some cases, complex organic compounds, such as potato extract, coconut milk and casein hydrolysate, have been added to the media.

According to Raina and Zapata (1997), the nitrogen composition of the culture medium plays a significant role in androgenesis. For example, decreasing the ammonium nitrate and increasing glutamine enhance embryo development in many cereal species. In addition to macro- and micro-nutrient, sugar and vitamins are required for anther culture basal media (Ellen, 1996). In most plant tissue cultures, the efficiency of photosynthesis is low due to limitation in carbon dioxide availability. Thus, sugar is required as source of energy for adequate growth. The most commonly used sugar for anther culture is sucrose although other sugars such as ribose, maltose and glucose have been found to be better than sucrose for some species (Sandra, 1996). Vitamins may be useful for anther culture to enhance growth significantly but at very low concentration. Of all vitamins, only thiamine ($B_1$) has been shown to be essential for many species of plant culture (Ellen, 1996). Murashige and Skoog medium (1962), as well as many others, also include nicotinic acid (niacin) and pyridoxine ($B_6$). Myo-inosytol is characterized as a vitamin, but it is a sugar alcohol, which can be added in the culture media to enhance membrane and cell wall development.

Another component of media for anther culture are plant growth regulators. Two familiar PGRs, namely auxins (NAA, 2,4-D, IAA and IBA) and cytokinins (BAP, BA, kinetin,) are most commonly used alone or in combination for anther culture. Auxins are used in induction of callus, root and shoot morphogenesis while cytokinins are used mostly to induce shoot bud induction, development and multiplication. Cytokinin often inhibits embryogenesis and root induction. Auxin are often used in combination with cytokinin, especially in species in which a callus phase is intermediate in the production of haploid plants (Nitsch, 1969). For organogenic culture system, a general rule is that an auxin: cytokinin ratio greater than 1 leads to root formation and an auxin: cytokinin ratio less than 1 results in shoot formation (Ellen, 1996).

### 2.10.3 Application of anther culture in crops

The most important application of anther culture is in obtaining of haploids or double haploids, which are useful in genetic studies and in plant breeding. Prior to the1960s, spontaneous interspecific hybridization and irradiated pollen technique were sources of haploids, but usually only infrequently and in a very small numbers (Sandra, 1996). The discovery of androgenic haploids of Datura (*Datura innoxia*) by Ghua and Maheshwari (1964) contributed for new methodology of developing haploids from male gamete or anther. This work was rapidly extended using tobacco (*Nicotiana tabacum* L.), which became the model species for anther culture experiment.

The theoretical consequences and practical application of double haploid systems depend on the breeding system of the crop in question weather it is predominately self- or cross-pollinating (Snape, 1989). In self-pollinating species, where a cultivar usually consists of single homozygous genotypes, there is the potential to use these systems to directly generate new cultivars. Conventional breeding combines useful traits, like better productivity or adaptation, by crossing different varieties. In the case of autogamous species, releasing a new cultivar depends on genetic homozygosity. Creation of homozygous plant by conventional breeding method, however, requires seven to nine generations, after the original cross. Double haploids enable the breeder to develop complete homozygosity from heterozygote parents in a single generation and thus speed up the procedure of new cultivar development. Successful cultivars of wheat, rice, maize and barely have been developed using this approach (Kasha and Reinberg, 1981 cited from Likyelesh Gugsa, 2005). In addition to direct use of as a potential varietal material, double haploids with self-pollinating crops can serve as intermediate parents for further crosses or as parents for $F_1$ hybrid, if a suitable system is available.

In cross-pollinating species, double haploids have an advantage in improving selection efficiency for quantitative characteristics by inserting them in each cycle of recurrent selection scheme. Although a generation of haploidization and chromosome doubling slow down each breeding cycle, such situation can be compensated by the increase in selection efficiency, and efficiency comparison with conventional scheme should consider the rate of response per year as well as the magnitude of response per cycle. In addition, since many cross-pollinating species are dioecious or have self-incompatibility mechanism that prevents the easy development of inbred lines, double haploids can be used in production of homozygous lines directly from selected parents. Such lines can be evaluated for specific combining abilities, and hence form hybrids or synthetic cultivars seed that provide a uniform and more acceptable product (Snape, 1989). Although the issue of inbreeding depression restricts the usefulness of double haploid in out-crossing population, if this is not a major problem, then double haploids may open the avenue to homozygous cultivars that perform equal to the best hybrids or population of out-crossing species (Griffing, 1975).

The out-crossing and self-incompatibility nature of niger make breeding efforts stagnant and thus the application of anther culture for niger improvement is very important. Some studies related to anther culture of niger have been conducted for Indian genotypes. Embryogenesis and organogenesis from anther culture of six Indian genotypes were studied in five different basal media (Sarvesh *et al,* 1993a). From this study, it was found that both embryogenic and non-embryogenic callus induced on the above basal media containing different combination and concentration of 2,4-D/NAA and kinetin. Embryogenic callus developed into embryo in Chaleff's R-2 media containing different concentrations of kinetin and NAA while non-embryogenic callus were differentiated into shoot in Murashige & Skoog (MS) media supplemented with 1-2 mg/l BAP and 0.1-0.2 mg/l NAA. Embryoids developed from embryogenic callus and shoot differentiated form non-embryogenic callus were successfully grown into plantlets upon transferring them onto Chaleff's R-2 and MS media devoid of growth regulators.

Haploid plant of niger have also been derived from nine different Indian genotypes to study androclonal variation (Sarvesh *et al,* 1994b). According to this study, one hundred and fifty plants obtained from anther callus of the niger genotype Ootacamund were successfully transferred to pots and grown to maturity. Out of these 150 plants, 8 plants were fertile and found to be diploid (2n = 30) and showed significant variations in agronomical characters like plant height, leaf length, and size of capitulum in both first and second generations. Dwarfs, larger flower head types and self-compatible plants recovered in this study are extremely useful for the improvement of niger. This successful achievement of anther culture for Indian genotypes might be applicable for Ethiopian genotype where the crop is found in large diversity. Since haploid plant production from anther culture is genotype and environment dependent it is necessary to investigate anther culture for Ethiopian genotypes that this paper presents.

# 3. OBJECTIVES

## 3.1 General objective

The general objective of the thesis was to regenerate plants from anther culture of Ethiopian niger (*Guizotia abyssinica* (L.f) Cass.)

## 3.2 Specific objectives

The specific objectives of this study are:-

(i) To identify the appropriate harvesting stage of capitula (buds) for anther culture.

(ii) To optimize the appropriate duration of cold-pretreatment of capitula (buds).

(iii) To study the effects of genotypes and basal media for callus induction in anther culture of niger.

(iv) To investigate the effects of different plant growth regulators for callus induction.

(v) To study the effects of different basal media and plant growth regulators for regeneration.

(vi) To evaluate the survival rate of plantlets in glasshouse.

## 4. MATERIALS AND METHODS

### 4.1 The donor plants material and growth conditions

Four varieties of niger namely Fogera, Esete, Kuyu and Shambu were taken as a plant materials for this study (Table 1.). Seeds of these varieties were obtained from Holetta Agricultural Research Center (HARC), Highland Oil Crops Research Division. The anther donor plants were raised in a pot containing a soil composed of red soil, compost and sand at a ratio of 3:2:1 respectively and allowed to grow in glass house, at HARC, at average temperature of $25^0$ C ± $2^0$ C under natural light condition.

**Table1. Seed yield, oil content and some agronomic characteristics of niger varieties used for the present study.**

| Variety | Seed yield (Kg/ha) | Oil content (%) | Maturity (days) | Plant height (cm) |
|---|---|---|---|---|
| Fogera | 820 | 41 | 146 | 138 |
| Esete | 830 | 39 | 146 | 139 |
| Kuyu | 1060 | 41 | 138 | 131 |
| Shambu | 970 | 39 | 136 | 130 |

Source: Highland Oil Crops Progress Report, EIAR, Holetta Center, 2007.

### 4.2 Identification of the appropriate harvesting stage of capitulea (buds)

External staging system of capitula (buds) was undertaken in order to determine the appropriate stage of capitula where maximum number of responsive anthers can be isolated. Capitula (buds) of the variety shambu were categorized into four stages based on the extent of opening of sepal and emergence of petals within flower head. Twelve samples of capitulea from each stage were taken and their diameter was measured using caliper. The capitulea diameter of each stage was determined using interval scale. Finally, the state of disc floret at each stage was characterized in terms of floret and anther colour at three different position of capitulea (i.e., center, medium and outer).

## 4.3 Bud (capitulae) collections and surface sterilization

Freshly harvested, immature flower buds were collected from glass house grown plants. Capitulea were surface sterilized in a laminar flow cabinet with 0.1% (m/v) mercuric chloride for 8 minutes and rinsed three times with sterilized distilled water.

## 4.4 Anther excision and plating

Young florets containing the anthers with pistil were excised aseptically from the immature capitulum and placed under stereomicroscope. Using fine blades, the basal portion of the floret was cut and the five fused anthers were isolated using fine needle without causing damage. Ten anthers were cultured in each test tube (25 x 150 mm) or petri dish (80 mm diameter) containing 15 ml basal media supplemented with different concentration and combination of auxin and cytokinin.

## 4.5 The effect of cold pre-treatment duration on callus induction

Six immature buds were taken from the 1$^{st}$ and 2$^{nd}$ stages of capitula to study the effect of cold pre-treatment on the induction of callus from anther of variety Shambu. The immature flower buds were pre-treated at $4^0$C for variable durations (12, 24, 36 and 48 hours duration). Ten anthers, from each pre-treated flower buds, were cultured in test tubes containing MS basal media supplemented with 2 mg/l 2, 4-D and 0.3 mg/l kinetin replicated three times. To confirm the effect of cold pre-treatment, anthers from the flower buds without cold pre-treatment (control) were also cultured in the same way. The frequency of responsive anthers was assessed in terms of callus induction percentage.

## 4.6 Effect of genotypes and basal media on callus induction

Four basal media namely MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968), N6 (Chu, 1978) and LS (Linsnaier and Skoog, 1965) supplemented with 2 mg/l 2,4-D plus 0.3 mg/l kinetin and 2mg/l NAA plus 0.3 mg/l kinetin were investigated for callus induction using anthers of the four varieties stated in section 3.1. A test tube with ten anthers was a unit of replication. Anthers from each variety were cultured with four replications for each medium with two types of PGRs combinations stated above. All media were supplemented with 2% sucrose and solidified with 0.8% agar throughout. After two weeks time of the above culture conditions, NN (Nitsch and Nitsch, 1969) medium were also tested for callus induction.

## 4.7 Effects of different concentrations and combinations of 2, 4-D and kinetin on callus induction

Anthers from Shambu and Fogera were plated in test tubes containing MS medium supplemented with ten different combinations of 2, 4-D and KN (Table 2). In both cases a test tube with ten anthers was a unit of replication and there were three replications for each treatment.

## 4.8 Culture conditions and data recording

Anthers were excised from the appropriate harvested stages of capitula (Stage 1 and Stage 2) which were cold-pretreated at $4^0$ C. All culture tubes and dishes were incubated in moist chamber under 16 h light (2000lux fluorescent intensity) and 8 h dark cycle at $22^0$C± $2^0$ C. The response of anthers for callus formation was observed every week and the numbers of anthers that induce callus in each test tube and petridsh were counted. Induced callus of each variety was expressed as a percentage. Sub-culturing was carried out every two weeks.

**Table 2. The different 2, 4-D and KN concentration and combination used to test the effect of PGRs for callus induction on MS medium**

| Treatments | Replications | Plant growth regulators | |
|---|---|---|---|
| | | 2,4-D (mg/l) | Kinetin (mg/l) |
| **A** | 3 | 0.5 | 0.0 |
| **B** | 3 | 1 | 0.1 |
| **C** | 3 | 1 | 0.3 |
| **D** | 3 | 1 | 0.5 |
| **E** | 3 | 2 | 0.1 |
| **F** | 3 | 2 | 0.3 |
| **G** | 3 | 2 | 0.5 |
| **H** | 3 | 3 | 0.1 |
| **I** | 3 | 3 | 0.3 |
| **J** | 3 | 3 | 0.5 |

### 4.9 Types of calli induced from anther culture of niger

After the preliminary observation trial that helps to select the best responsive varieties and appropriate media for callus induction, experiments were conducted using only two varieties (Shambu and Fogera) and the two selected media (MS and $B_5$) in addition to NN (Nitsch and Nitsch, 1969) medium. All basal media were supplemented with 2 mg/l NAA plus 0.3 mg/l kinetin. Ten anthers of Shambu and Fogera were cultured into the Petri dish containing the above basal media each replicated four times. All cultures were maintained in moist chamber under 16 h light (2000lux fluorescent intensity) and 8 h dark cycle at $22^0C \pm 2^0$ C.

### 4.10 Shoot regeneration from anther-derived calli of niger

#### 4.10.1 Effect of basal media on shoot regeneration

Four to five weeks old calli induced from anthers of Shambu and Fogera in MS, $B_5$ and NN media were transferred onto MS-regeneration medium supplemented with 1 mg/l BAP and 0.2 mg/l NAA. Three calli (2 embryogenic plus one non-embryogenic) with size measured approximately 3-5mm were transferred in plastic jar containing 30ml of regeneration medium. The cultures were maintained in the same condition as for callus induction experiments (16 h / 8 h light and dark cycles) and each medium was replicated three times. The number of calli that developed shoots until 3 weeks were recorded. Percentages of shoot formation from each source media were calculated. The experiment was repeated twice under the same conditions.

#### 4.10.2 Effect of different concentrations and combinations of kinetin and IAA for shoot regeneration

After the observation of preliminary results of shoot regeneration, an experiment was conducted to test the effect of different concentrations and combinations of PGRs for shoot regeneration. Embryogenic calli of the variety Shambu derived from MS medium supplemented with 2 mg/l 2,4-D and 0.3 mg/l KN was transferred into MS-regeneration media supplemented with three levels of kinetin (1, 2, 3 mg/l) alone or in combination with IAA (0.5 and 1mg/l) (Table 3). A jar with a single callus, measured approximately 2-3 mm diameter, was a unit of replication and there were six replications for each treatment. The cultures were incubated at $22^0C\pm 2^0$ C with 16 h photoperiod in moist chamber. The number of cultures with shoot was recorded for 3 weeks. The experiment was repeated twice in six replications.

**Table 3. The different KN and IAA concentration and combination used to test the effect of PGRs for shoot regeneration on MS medium.**

| **Treatments** | Replications | **Plant growth regulators** | |
|---|---|---|---|
| | | **KN (mg/l)** | **IAA (mg/l)** |
| **A** | 6 | 1 | 0.0 |
| **B** | 6 | 1 | 0.5 |
| **C** | 6 | 1 | 1.0 |
| **D** | 6 | 2 | 0.0 |
| **E** | 6 | 2 | 0.5 |
| **F** | 6 | 2 | 1.0 |
| **G** | 6 | 3 | 0.0 |
| **H** | 6 | 3 | 0.5 |
| **I** | 6 | 3 | 1.0 |

### 4.11 Rooting experiment

Rooting experiment was conducted using shoots of both varieties on MS-rooting media supplemented with various concentrations of IBA. The treatments were 0.5, 1, 2mg/l IBA and the control (without IBA). A culture with single shoot was used as a unit of replication and there were 4 replications for each variety. The cultures were maintained in moist chamber of 16 h photoperiod at $22^0$C± $2^0$ C. Percentage of cultures with root and the numbers of roots produced per shoot were recorded in every three days.

### 4.12 Acclimatization and growing of plantlets in the glasshouse

Plantlets with the approximate height of 5-8 cm, with well developed roots of 2-3 cm length and 2-3 leaves, were removed from the culture jars using pincers and transferred into pots. The pots were filled with red soil, compost and sand in the ratio of 3:2:1, respectively. For acclimatization, each pot was covered with plastic bags and kept in the growth room at $22^0$C-$25^0$C ± $2^0$ C with photoperiod of 16 h and light intensity 2000lux.

After two weeks, pots were transferred to glass house, the plastic bag gradually removed and regenerants were grown to maturity.

### 4.13 Experimental design and data analysis

All culture experiments were replicated and laid out in completely randomized design (CRD). Analysis of variance (ANOVA) was conducted using SAS computer software (Version, 1998). The mean separation method (LSD) was used to compare means between treatments.

# 5. RESULTS

## 5.1 Determination of the appropriate harvesting stage of capitula (buds)

A workable morphological staging system of capitula (buds) was identified in order to determine the stage of capitula with maximum number of responsive anthers (Fig. 1). According to this staging system, disc florets taken from Stage 1 and Stage 2 of capitula were found to be stages with maximum number of responsive anthers. However, the responsiveness of anthers at these stages varies with the position of florets. Florets of Stage 1 capitula at the center position were very small, too young and with sticky anthers and those of Stage 2 at the outer position (near the sepal) were old enough with some pollen released anthers and thus non-responsive anthers could be isolated at these positions (Appendix I, Table 1). It was observed that Stage 3 and Stage 4 of capitula were considered as stages with least number of responsive anthers since almost all or the majority of the disc floret at these stages contained matured anthers that release pollen and such anthers are usually considered as too old for androgenesis (Appendix I Table 1). In general, Stage 1 capitula with florets at medium and outer and Stage 2 capitula with florets at center and medium were determined as optimum stages with florets possessing maximum number of responsive anthers. It was also observed that best responsive anthers in these florets identified by their whitish green or greenish yellow colour.

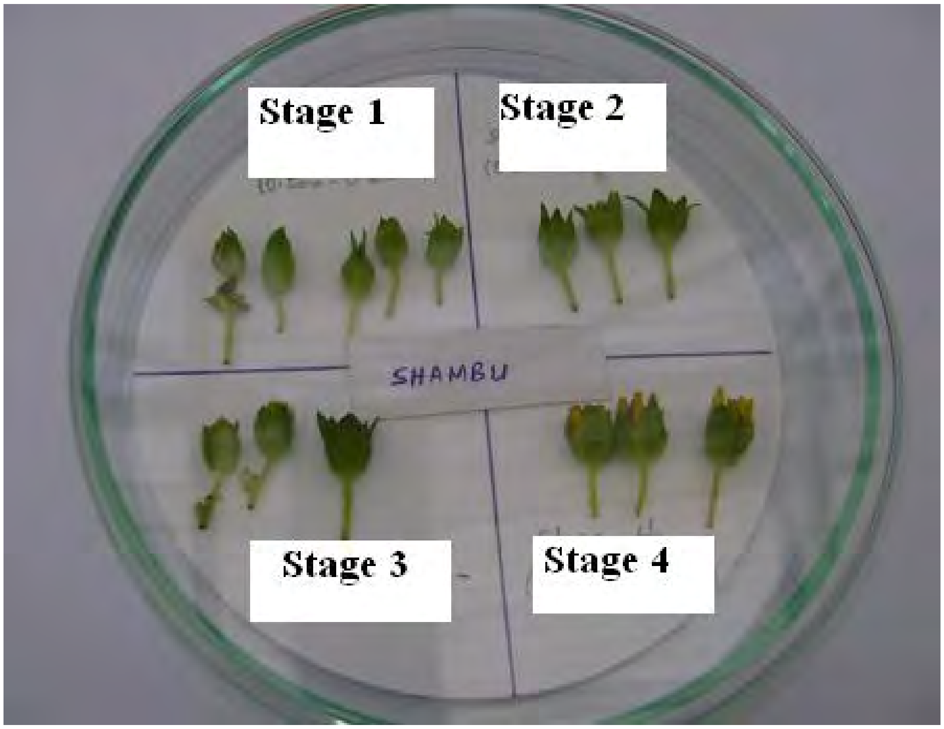



Fig 1. The different stages of capitula in niger, variety Shambu. Stage 1: capitula fully covered by sepal, Stage 2: Capitula covered with slightly opened sepal, Stage 3: capitula covered with opened sepal and greenish yellow petal emerged with sepal and Stage 4: capitula covered with opened sepal and yellow petal emerged with sepal.

### 5.2 Effect of cold-pretreatment on callus induction in anther culture of niger

The effect of cold pretreatment on callus induction in anther culture of the variety Shambu was presented in the Appendix I (Table 2) and in the text (Fig. 2). The result indicated that the least callus induction percentage was obtained from anthers taken from capitula without cold-pretreatment. Callus induction percentage increased by 43% when capitula were pretreated for 12 h time duration. The highest callus induction percentages 70% and 76.7% were obtained from anthers pretreated for 24 hours, which were taken from Stage 1 and Stage 2 capitula, respectively. In the second best cold-pretreatment duration (36 h), the same percentage of callus induction percentage (60%) was obtained for both stages. The callus induction percentages of anthers taken from 48 h pretreated capitula were 12% (for Stage 1) and 13% (for Stage 2). Based on this specific investigation, the best cold-pretreatment duration of capitula (buds) for better induction of callus in anther culture of the variety Shambu was 24 hours.

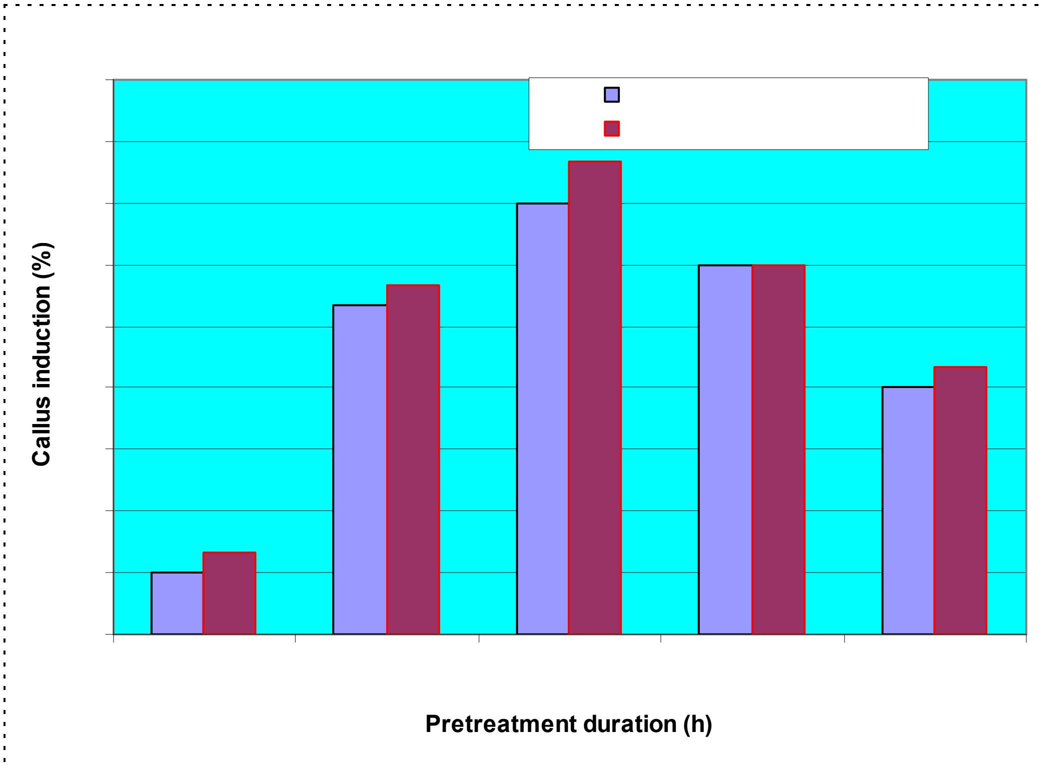



Fig 2. The effect of cold-pretreatment ($4^0$C) duration on callus induction in anther culture of the variety Shambu

### 5.3 Effect of genotypes and culture media on callus induction

Results of the preliminary callus induction experiment were presented in the appendix I (Table 3a - 3d) and in text (Table 4). Based on these results, it was found that anthers from variety Shambu followed by Fogera showed better callus induction percentage in all tested culture media supplemented with either 2 mg/l 2,4-D + 0.3 mg/l KN or 2 mg/l NAA+ 0.3mg/l KN. These varieties gave better callus induction percentage on $B_5$ and MS culture media than on $N_6$ and LS media. Variety Shambu showed highest callus induction percentage on $B_5$ (42.1%) followed by MS (36%) culture media both supplemented with 2 mg/l 2, 4-D and 0.3 mg/l KN. The second best responsive variety Fogera gave 34.2% and 28.9% on $B_5$ and MS media respectively, supplemented with the same PGRs used in variety Shambu. The callus induction percentage of the remaining two varieties namely Kuyu and Esete were much lower than Shambu and Fogera in all culture media supplemented with both types of PGRs (Table 4). The two varieties, however, were not significantly (p=0.05) varied in callus induction percentage in both treatments (Table 4). The above results indicated that Shambu and Fogera were considered as the most responsive varieties for callus induction in anther culture of niger and selected for further anther culture experiment.

Percentage of callus induction among the four varieties cultured on MS medium supplemented with 2 mg/l 2, 4-D and 0.3 mg/l KN ranged between 12.8% for variety Kuyu and 36.8% for variety Shambu with highest (23.5%) average callus induction percentage (Table 4). The second best fit medium $B_5$, with the same combination of PGRs, gave an average of 22.2% of callus induction, with maximum of 42.1% for variety Shambu and a minimum of 5.1% for variety Kuyu (Table 4). The maximum, minimum and average callus induction percentage of $N_6$ (23.7%, 2.6%, 11.7%) and LS (5.3 %, 15.0%, 8.8 %) with the same PGRs combinations stated above were significantly (p=0.05) lower than MS and B5 basal media (Table 4). Similar interpretations can be given to callus induction percentage of the basal media supplemented with 2 mg/l NAA and 0.3 mg/l KN. Based on this result, MS basal media followed by $B_5$ was taken as efficient basal media for callus induction in anther culture of niger and these media were also used for further anther culture experiment.

Analysis of variance (Table 5) showed a significant (p=0.05) effect of culture media and genotype on the callus induction percentage of cultured anthers of niger (Table 4). However, the two factors affect callus induction independently since the interaction of media and genotypes was not significant at p=0.05

**Table 4. Percentage of callus induction during anther culture of four niger varieties in four different culture media supplemented with 2, 4-D (2mg/l) + KN (0.3mg/l) and NAA (2mg/l) + KN (0.3mg/l)**

| Varieties | Culture media | | | | **Mean** |
|---|---|---|---|---|---|
| | MS | $B_5$ | $N_6$ | LS | |
| | ***Treatment [1]*** | | | | |
| Shambu | 36.8 | 42.1 | 23.7 | 15.0 | **29.4*a*** |
| Kuyu | 12.8 | 5.1 | 7.9 | 5.0 | **7.7*c*** |
| Esete | 15.4 | 7.5 | 2.6 | 5.3 | **7.7*c*** |
| Fogera | 28.9 | 34.2 | 12.5 | 10.0 | **21.4*b*** |
| **Mean** | **23.5*A*** | **22.2*A*** | **11.7*B*** | **8.8*B*** | |
| | ***Treatment [2]*** | | | | |
| Varieties | MS | $B_5$ | $N_6$ | LS | **Mean** |
| Shambu | 30.0 | 35.0 | 21.1 | 13.2 | **24.8*a*** |
| Kuyu | 17.5 | 12.5 | 7.7 | 2.6 | **10.0*b*** |
| Esete | 13.2 | 2.5 | 2.5 | 7.5 | **6.4*b*** |
| Fogera | 27.5 | 33.3 | 10.0 | 7.5 | **19.6*a*** |
| **Mean** | **22.1*A*** | **20.8*A*** | **10.3*B*** | **7.7*B*** | |

***Treatment [1]*** Callus induction % for basal media supplemented with 2 mg/l 2,4-D + 0.3 mg/l KN
***Treatment [2]*** Callus induction % for basal media supplemented with 2 mg/l NAA + 0.3 mg/l KN
Means with the same letter along the row (upper case) and along column (lower case) are not significantly differ at p= 0.05

**Table 4. Analysis of variance for the effect of genotypes and culture media on callus production from anthers of four niger varieties.**

| Source of variance | Callus induction (%) | | |
|---|---|---|---|
| | DF | MS | F-value |
| | *Treatment 1* | | |
| Genotype | 3 | 1902.79 | 4.59*** |
| Culture medium | 3 | 893.67 | 9.30*** |
| Genotype x medium | 9 | 1.72 | 0.11 |
| Error | 45 | 96.1 | |
| | *Treatment 2* | | |
| Genotype | 3 | 1238.52 | 15.68*** |
| Culture medium | 3 | 766.64 | 9.7*** |
| Genotype x medium | 9 | 169.36 | 2.14 |
| Error | 45 | 78.99 | |

***Treatment 1*** Callus induction % for basal media supplemented with 2 mg/l 2, 4-D + 0.3 mg/ lKN
***Treatment 2*** Callus induction % for basal media supplemented with 2 mg/l NAA + 0.3 mg/ lKN
* * * = significant at p= 0.001

**5.4 Effects of different concentrations and combinations of 2, 4-D and kinetin for callus induction**

Results for the response of anthers of Shambu and Fogera at different concentrations and combinations of 2,4-D and KN were presented in the appendix I (Table 4a and 4b) and in the text (Fig. 3). According to this result, the two varieties failed to induce callus when lower concentration of 2,4-D (0.5mg/l) was used alone. Although the variety Shambu showed better callus induction percentage in every 2,4-D and KN combinations as compared to the variety Fogera the variation between them was minimal (Fig. 3). The variation in callus induction response was higher among the different level of 2,4-D and KN than among the same level of 2,4-D with different level of KN. Callus induction percentage became maximum at Treatment F and began to decline at Treatment G and thus Treatment F (2mg/l 2, 4-D + 0.3 mg/l KN) was taken as the best PGRs combination for callus induction in anther culture of Shambu and Fogera. Induced calli at Treatments (E, F G) were presented in the appendix II (Fig. 1)

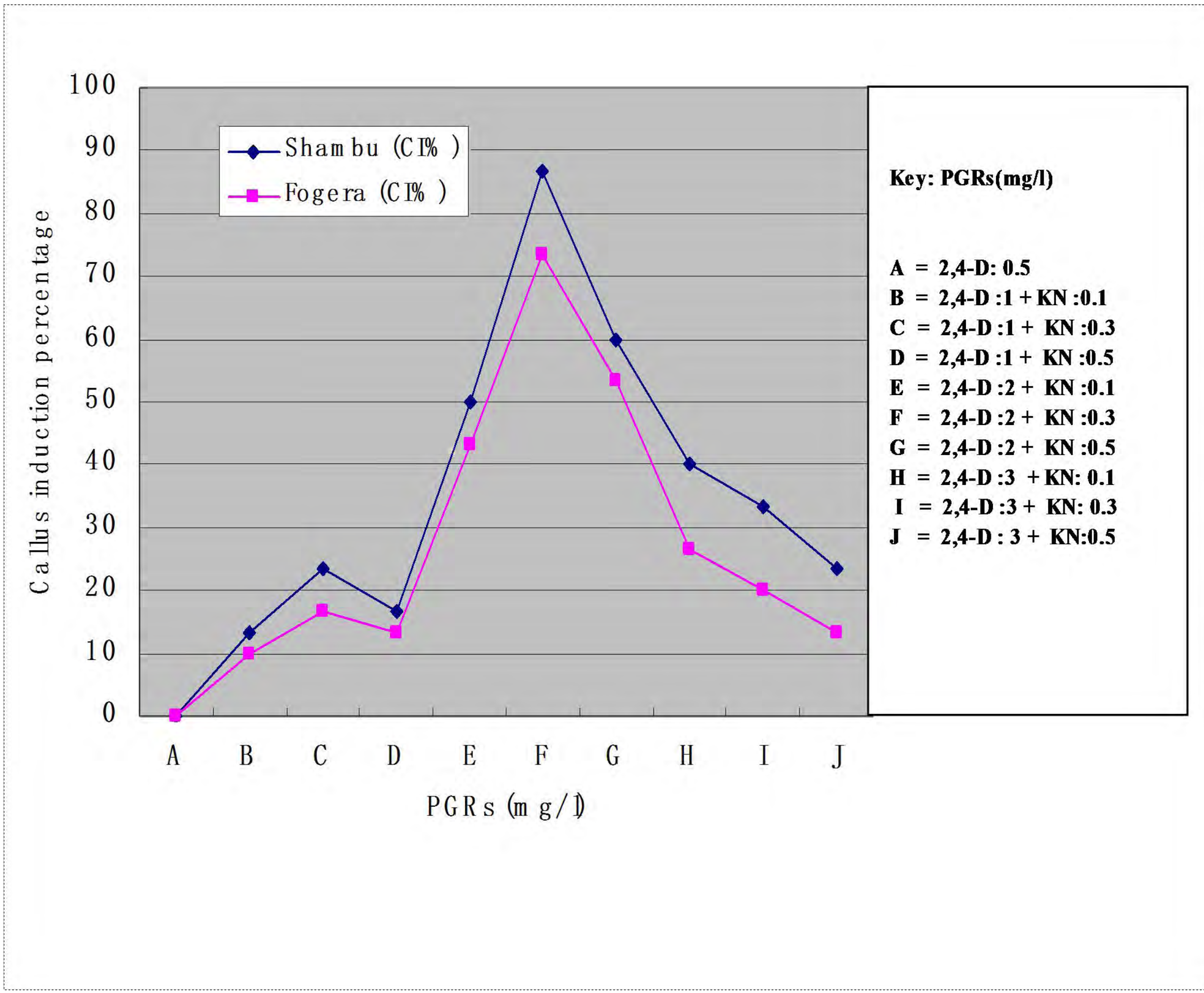



Fig 3. Callus induction percentage in anther culture of Shambu and Fogera at different concentration and combination of 2,4-D and KN.

## 5.5 Types of calli induced from anther culture of niger

Based on visual observation, two distinct callus types namely embryogenic calli (EC) and non- embryogenic calli (NEC) were recognized. NE calli were distinguished by their amorphous morphology and in some cases friable texture but their colour varied among the three media. In MS media NE calli were identified by their whitish- yellow colour while in $B_5$ and NN media, NE calli were distinguished by their brown greenish-yellow and brown whitish-yellow colour respectively (Fig. 4). Embryogenic calli were usually appeared as immature embryo with different texture and colour among the three media. In MS medium, such calli produced with compact texture and greenish-yellow colour (Fig. 5A and 5B). Embryogenic calli were mainly produced on NN with shiny and smooth texture and whitish at earlier stage and later whitish-green in colour (Fig. 5C). Embryogenic calli of B5 were found to be compact in texture and reddish- yellow in colour (Fig. 5D). Percentages of calli of each type were also varied among the three media. NE calli were dominantly produced in MS medium. Maximum percentage of NE calli (100%) was obtained in MS medium from anthers of Fogera (Table 6). High percentage of embryogenic calli (80%) was produced in NN medium from anthers of Shambu. It was also observed that embryogenic calli on NN medium showed better differentiation rate as compared to embryogenic calli on $B_5$ (Fig. 5 E and 5F).

Fig. 4 Non -embryogenic calli (NEC) induced from anthers of Shambu on three different media. (A) NEC on MS medium after four weeks of the initial culture, (B) NEC on $B_5$ medium after four weeks of the initial culture and (C) NEC on NN medium after three weeks of the initial culture

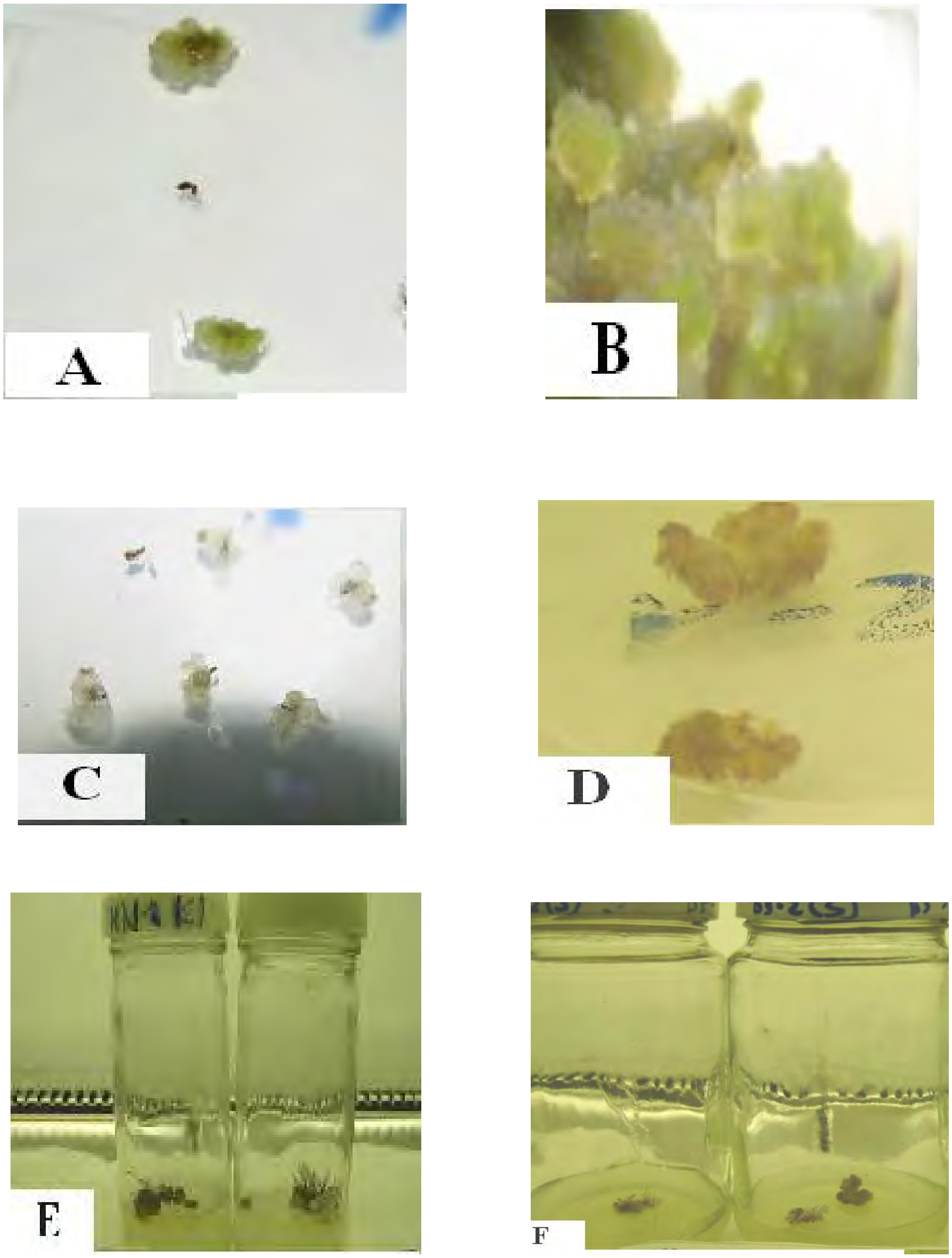



Fig 5. Embryogenic calli induced from anthers of Shambu in three different media. (A) EC induced on MS medium just two weeks after culture. (B) EC on MS medium four weeks after the initial culture, (C) EC induced on NN medium just two weeks after culture, (D) EC induced on $B_5$ medium after three weeks of the initial culture, (E) Differentiated calli on NN medium after four weeks of the initial culture and (F) Differentiated calli on $B_5$ after four weeks of the initial culture.

**Table 6. Percentage of embryogenic and non-embryogenic calli in anther culture of varieties Shambu and Fogera**

| Media | Variety | No of calli plated/ variety | No of calli responded | | Callus induction percentage | |
|---|---|---|---|---|---|---|
| | | | Embryogenic calli | Non-embryogenic | Embryogenic calli | Non-embryogenic |
| MS | Shambu | 40 | 8 | 32 | 20 | 80 |
| | Fogera | 40 | 0 | 40 | 0 | 100 |
| $B_5$ | Shambu | 40 | 16 | 24 | 40 | 60 |
| | Fogera | 40 | 16 | 24 | 40 | 60 |
| NN | Shambu | 40 | 32 | 8 | 80 | 20 |
| | Fogera | 40 | 24 | 16 | 60 | 40 |

### 5.6 Effect of culture media on shoot regeneration

Of the total of 108 calli transferred on MS- regeneration media, after 2 -3 weeks of transferring, 64 calli (59.3%) produced shoots (Appendix I, Table 5). Most of the shoots regenerated were derived from embryogenic calli of NN and $B_5$ media (Fig. 6 A and 6B). In some cases, shoots were regenerated from embryogenic calli of MS (Fig. 6C). Shoots from such calli, however, developed lately after being sub-cultured twice into fresh MS - regeneration media (Fig 6D).

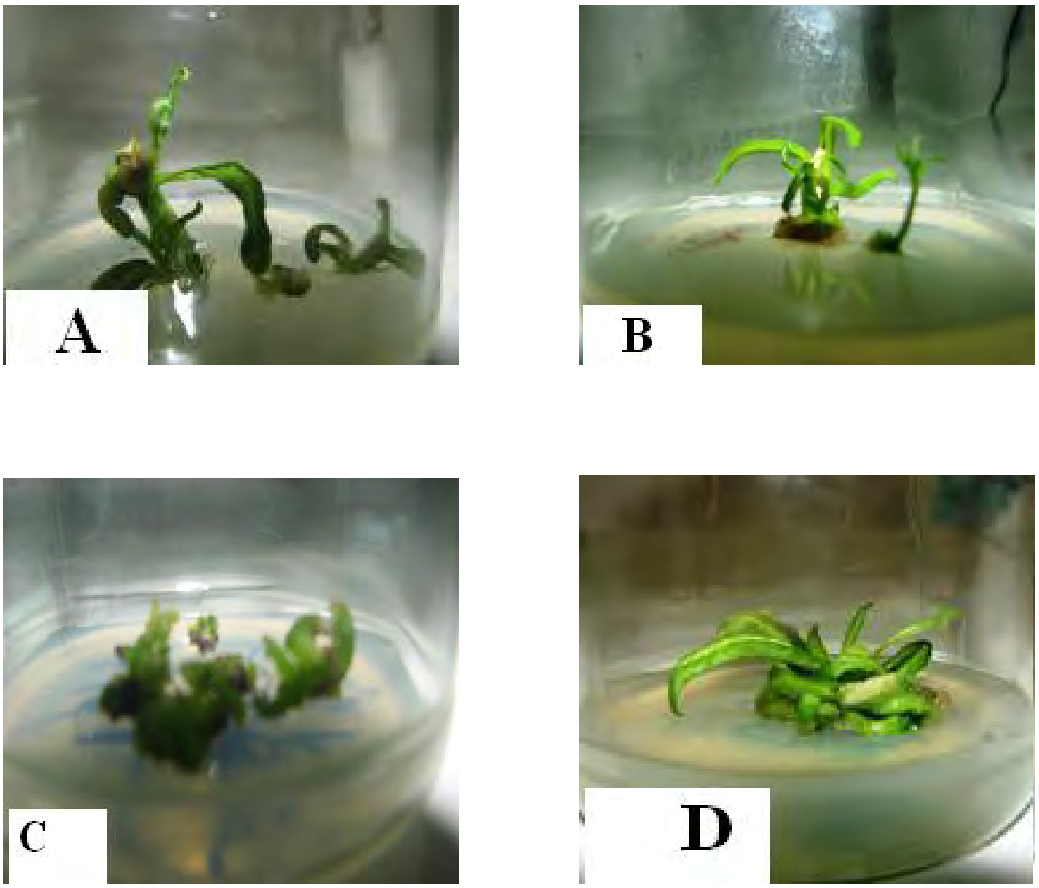



Fig 6. Shoot regeneration of anther-derived calli induced from different media
(A) Shoots developed from embryogenic calli of NN (var.Shambu), (B) Shoots developed from embryogenic calli of $B_5$ (var.Fogera) , (C) Immature shoots from embryogenic calli of MS (var.Shambu) , (D) Regenerated shoot from embryogenic calli of MS obtained after subculture (var.Shambu).

Calli transferred from NN medium to MS-regeneration medium were the best responsive calli followed by $B_5$ for shoot regeneration. Calli from NN medium produced the highest mean percentage of shoot regeneration, 83.4% (Table 7). An average of 61.2% shoot regeneration was determined for calli obtained from $B_5$. Calli from MS media gave the lowest shoot regeneration percentage.

**Table 7. Shoot regeneration percentage of callus derived from three basal media in anther culture of varieties Shambu and Fogera**

| | Culture media | | |
|---|---|---|---|
| Variety | MS | $B_5$ | NN |
| Shambu | 33.3 | 66.7 | 88.9 |
| Fogera | 33.3 | 55.6 | 77.8 |
| Mean | 33.3 | 61.2 | 83.4 |

Analysis of variance (Table 8) revealed that there was significant difference (p=0.05) among the culture induction media in shoot regeneration (Table 8). However, callus induction media expressed their effect on regeneration independent of the varieties since interaction between the two factors was insignificant. ANOV also indicated that no significant difference between the two varieties in shoot regeneration within the culture induction media.

**Table 8. Analysis of variance for the effect of callus induction media on shoot regeneration of niger varieties Shambu and Fogera**

| | Shoot regeneration percentage | | |
|---|---|---|---|
| Source of variation | DF | MS | F-value |
| Genotype | 1 | 61.75 | 0.16 |
| Callus induction media | 2 | 3024.53 | 7.65* |
| Genotype X media | 2 | 61.75 | 0.16 |

*= Significant at p=0.05

## 5.7 Effect of different concentrations and combinations of PGRs for shoot regeneration.

Of the total 108 calli transferred to regeneration media, 28 (26%) produced shoots at different concentration and combination of kinetin and Indole acetic acid (Appendix I, Table 6). Percentage of cultures with shoots ranged from zero to 83% and thus considerable variations existed among the different PGRs tested for shoot regeneration. Calli from Shambu was failed to regenerate shoots at 1 mg/l KN alone while calli began to regenerate at 1 mg/l concentration of KN when 0.5mg// IAA was added to the regeneration media (Fig. 7). Equal percentage of shoot regeneration (25%) was obtained in regeneration medium supplemented with 1mg/l KN + 0.5 mg/l IAA and with 3 mg/l KN alone. Similarly, variation was observed in shoot regeneration percentage in cultures with 3 mg/l KN + 0.5 mg/l IAA and with 3 mg/l KN + 1 mg/l IAA. Regeneration medium supplemented with 2 mg/l KN combined with IAA (0.5, 1 mg/l) gave better shoot regeneration percentage (Fig. 7). Regenerated shoots of various treatments of regeneration media were presented in (Fig. 8). Regeneration medium with 2 mg/l KN + 1mg/l IAA produced maximum shoot regeneration percentage (83.3%). This value was two fold higher than the shoot regeneration percentage in regeneration medium with 2 mg/l KN + 0.5 mg/l IAA.

The response of the different combinations and concentration of KN and IAA not only differ in percentage of shoot formation but also in quality of shoots they produced. Poor quality of shoots, with weak stand or with very small stalk, were produced in cultures with low concentration of KN (1 mg/l) and higher concentration of KN (3mg/l) that combined with 0.5 and 1.0 mg/l IAA (Fig. 8A and 8B). Good quality regenerated shoots, those with better stand and even branched stem, obtained in cultures supplemented with intermediate concentration of KN (2 mg/l) either alone or in combination with 0.5 and 1.0 mg/l IAA (Fig. 8C and 8D).

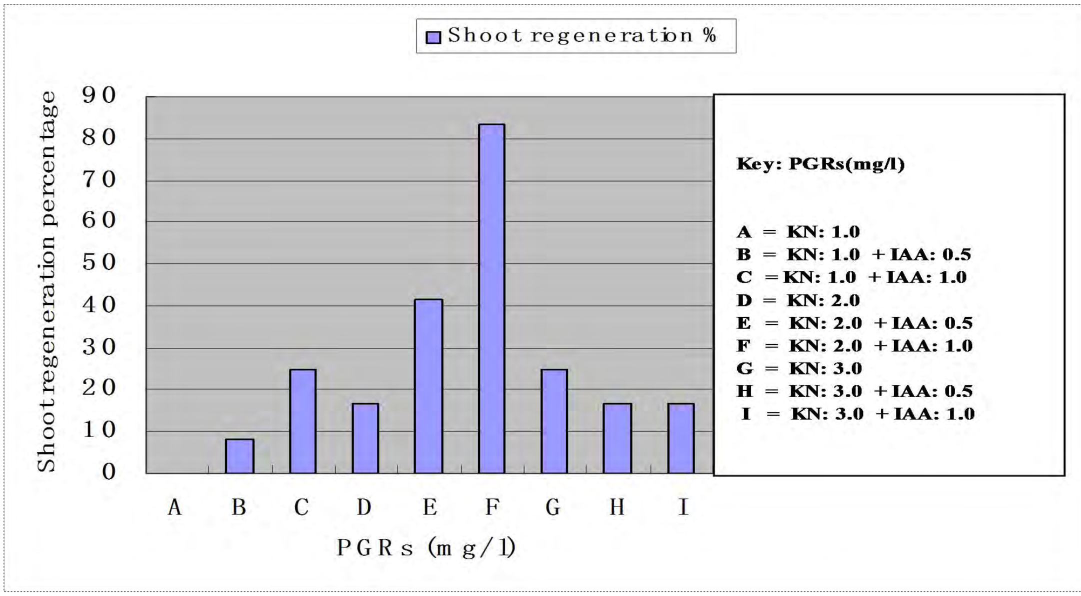
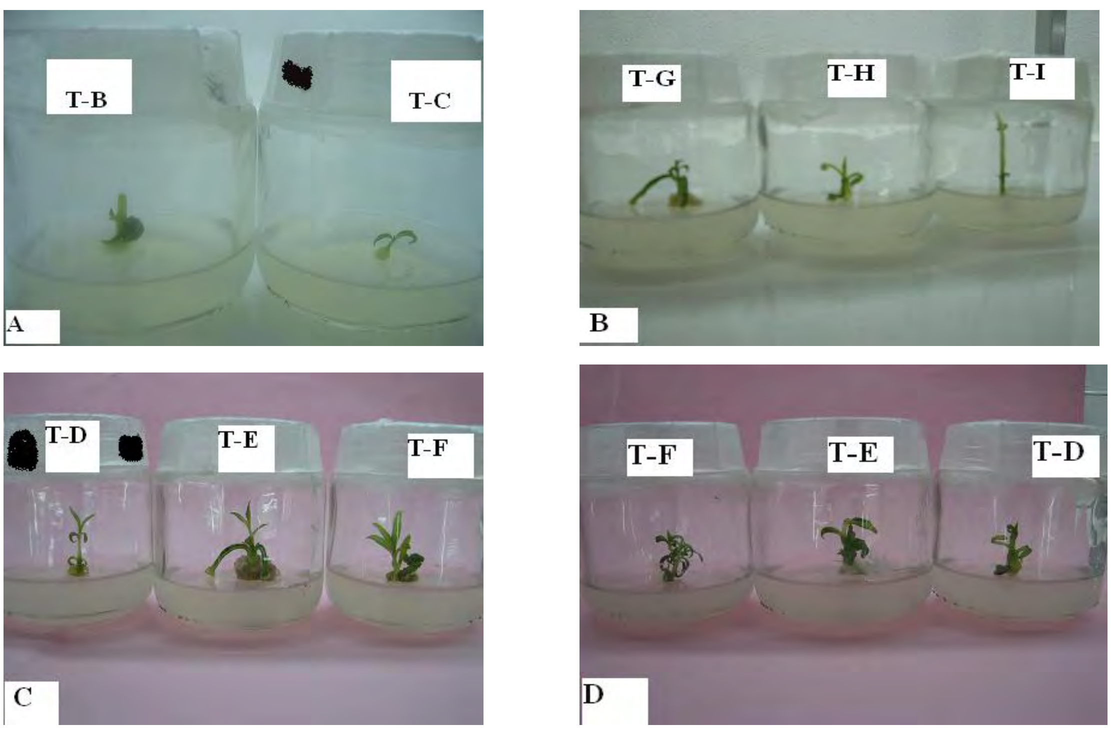



Fig 7. Shoot regeneration percentage in anther-derived callus of the variety Shambu at different concentration and combination of KN and IAA.




Note: T-B, T-C,T-D, T-E T-F, T-G, T-H, T-I refers to treatments that are assigned in Fig 8.

Fig 8. Shoots regenerated under the different KN and IAA concentration and combination. (A) and (B) shoots from calli of low regeneration potential observed in media supplemented with 1 mg/l KN + 0.5 mg/l IAA (T-B) and with 1 mg/l KN + 0.5 mg/l IAA (T-C) respectively. (C) and (D) Shoots from calli of good regeneration potential observed in media supplemented with 2 mg/l KN alone or in combination of 0.5, 1.0 mg/l IAA.

### 5.8 Rooting of shoots

The detailed data for every replicated culture of shoots was presented in the appendix I (Table 7). All MS - rooting media with three different concentration of IBA and control (0, 0.5, 1.0, and 2.0 mg/l) were able to produce roots for both varieties. Variety Shambu showed 100% rooting in all rooting medium treatments (Table 9). In the case of Fogera, however, the percentages of rooting at 1 mg/l and 2 mg/l IBA concentrations were 50% and 25% respectively. According to the results in (Table 9), there was no much difference among IBA concentrations except 2 mg/l for the average number of rooting. Visual observation of the rooting cultures, however, indicated that the media with 0.5 mg/l was the best rooting media since this media produced well-developed roots that enabled the shoots to grow faster (Fig. 9). Besides, this rooting media was also superior in average number of roots.

**Table 9. The effect of different concentrations of IBA for rooting percentage and number of roots produced**

| IBA (mg/l) | Genotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Shambu | | | | Fogera | | | |
| | No of shoots cultured for rooting | No of shoots produced roots | Percent-age of rooting | Av. No of roots per shoot | No of shoots cultured for rooting | No of shoots produced roots | Percent-age of rooting | Av. No of roots per shoot |
| 0.0 | 4 | 4 | 100% | 8.5 | 4 | 4 | 100% | 7.5 |
| 0.5 | 4 | 4 | 100% | 11.0 | 4 | 4 | 100% | 8.5 |
| 1.0 | 4 | 4 | 100% | 9.5 | 4 | 2 | 50% | 7.5 |
| 2.0 | 4 | 4 | 100% | 3.5 | 4 | 1 | 25% | 2.0 |

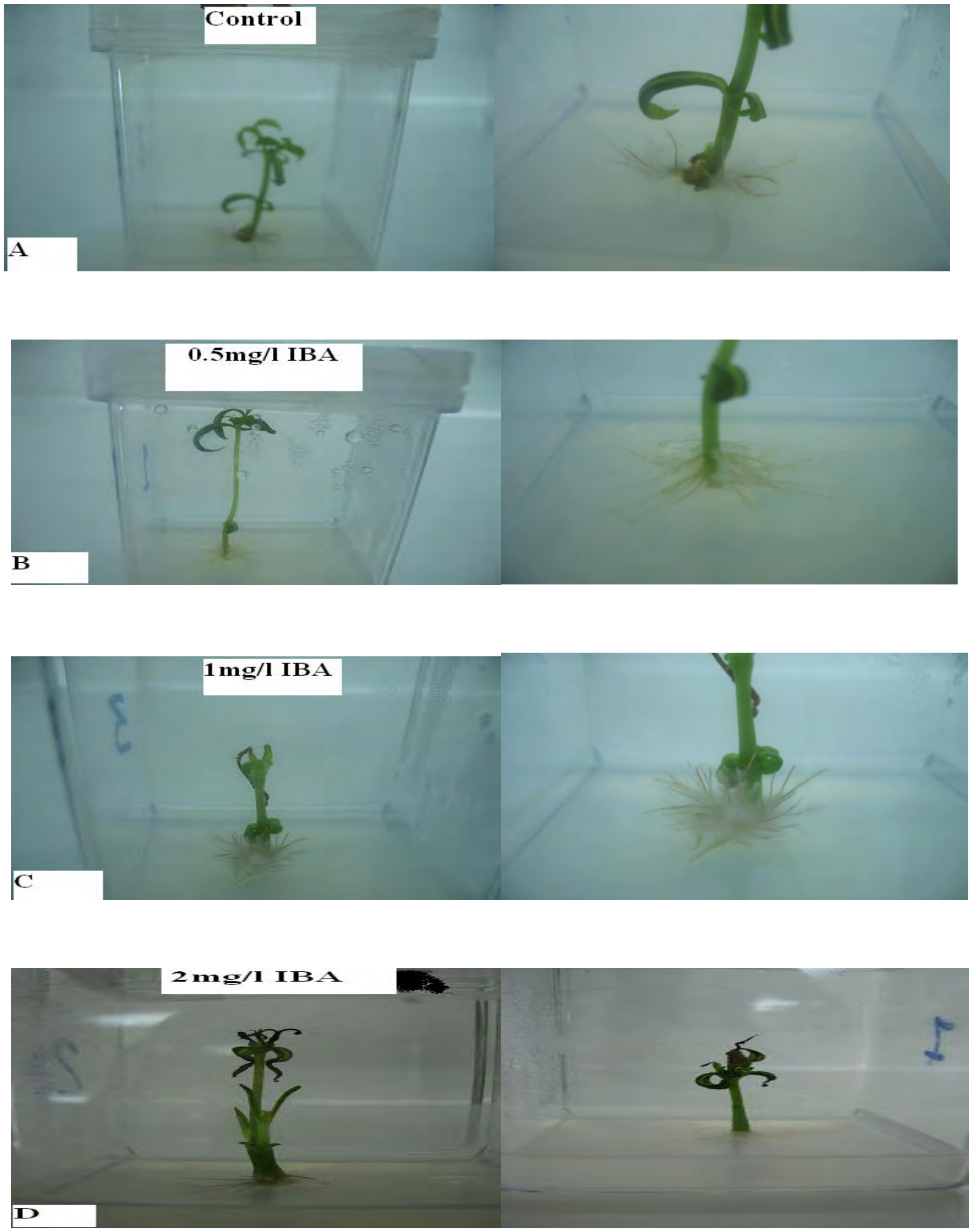



Fig 9. Root formation on MS-rooting medium supplemented with different concentration of IBA, incubated in moist chamber under 16 h light (2000lux fluorescent intensity) and 8 h dark cycle at $22^{0}C \pm 2^{0}$ C. (A) Shoot of Shambu placed on MS- rooting medium without IBA (with some roots and shoot elongation). (B) Shoot of Shambu transferred on 0.5 mg/l MS-rooting medium supplemented with 0.5 mg/l IBA (with better roots formation and shoot elongation). (C) Shoot of Shambu transferred on MS-rooting medium supplemented with 1 mg/l IBA (many roots and poor elongation of shoot). (D) Shoot of Shambu transferred on MS-rooting medium supplemented with 2 mg/l IBA (with very few roots and abnormal growth of shoot).

### 5.9 Acclimatization and growing in glasshouse

The number of regenerants transferred in the pot, the survival rate growthroom and glasshouse were presented in table 10. Sixteen Shambu and eleven Fogera regenerants were successfully transferred into pots and taken into the growthroom covered with plastic for acclimatization. However, 6 of regenerants of each variety died within a week due to fungi contamination of the soil (Appendix II, Fig.2). The remaining 10 Shambu and 5 Fogera regenerants were grown in the growthroom (Fig. 10) until two weeks and taken to glasshouse for further growth. In the glass house, 4 regenerants of Shambu and 3 regnerants of Fogera were wilted and died (Appendix II, Fig.2) and thus only 6 regenerants of Shambu and 2 regenerants of Fogera remained in the glasshouse (Table 10). Regenerants of Shambu and Fogera that survived in glasshouse are presented in Fig. 11. Pictures showing the growing condition of niger regenerants at different time were also presented (Fig. 12).

**Table 10. Number of regenerants transferred into pots, and their subsequent status under growthroom [16 h light (2000lux) and 8 h dark cycle] and glasshouse (under natural light and at average temperature of $25^0$ C ± $2^0$ C) conditions.**

| Varieties | Regenerants status | | | | |
|---|---|---|---|---|---|
| | No of plantlets transferred into pots | No of regenerants died in growthroom | No of regenerants survived in growthroom | No of regenerants died in glasshouse | No of regenerants survived in glasshouse |
| Shambu | 16 | 6 | 10 | 4 | 6 |
| Fogera | 11 | 6 | 5 | 3 | 2 |
| | Total= 27 | Total= 12 | Total= 15 | Total= 7 | Total= 8 |

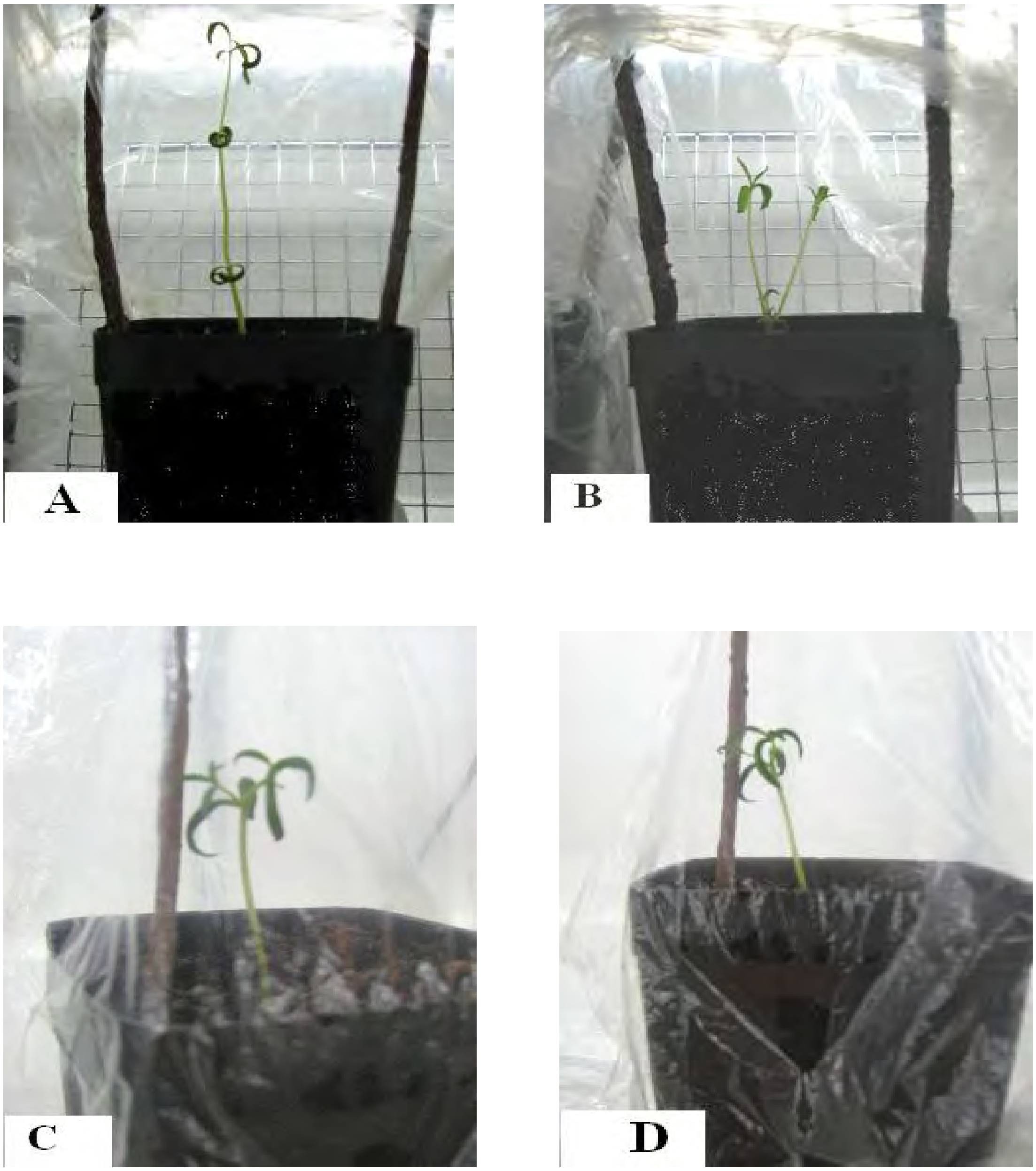



Fig 10. Regenerants of niger in the growthroom. (A&B) Regenerants of Shambu from anthers induced callus on NN medium supplemented with 2 mg/l 2,4-D + 0.3 mg/l KN, regenerated on MS – regeneration medium supplemented with 2 mg/l KN + 1 mg/l IAA and MS- rooting medium supplemented with 0.5 mg/l IBA. (C&D) Regenerants of Forgra induced callus on $B_5$ medium supplemented with 2 mg/l 2,4-D + 0.3 mg/l KN and regenerated shoot and root on the same media compositions of the regenerants of the above variety ( Shambu). Plantlets were grown under growthroom conditions [16 h lights (2000lux) and 8 h dark cycle and a temperature of $22^0$C-$25^0$C ± $2^0$ C].

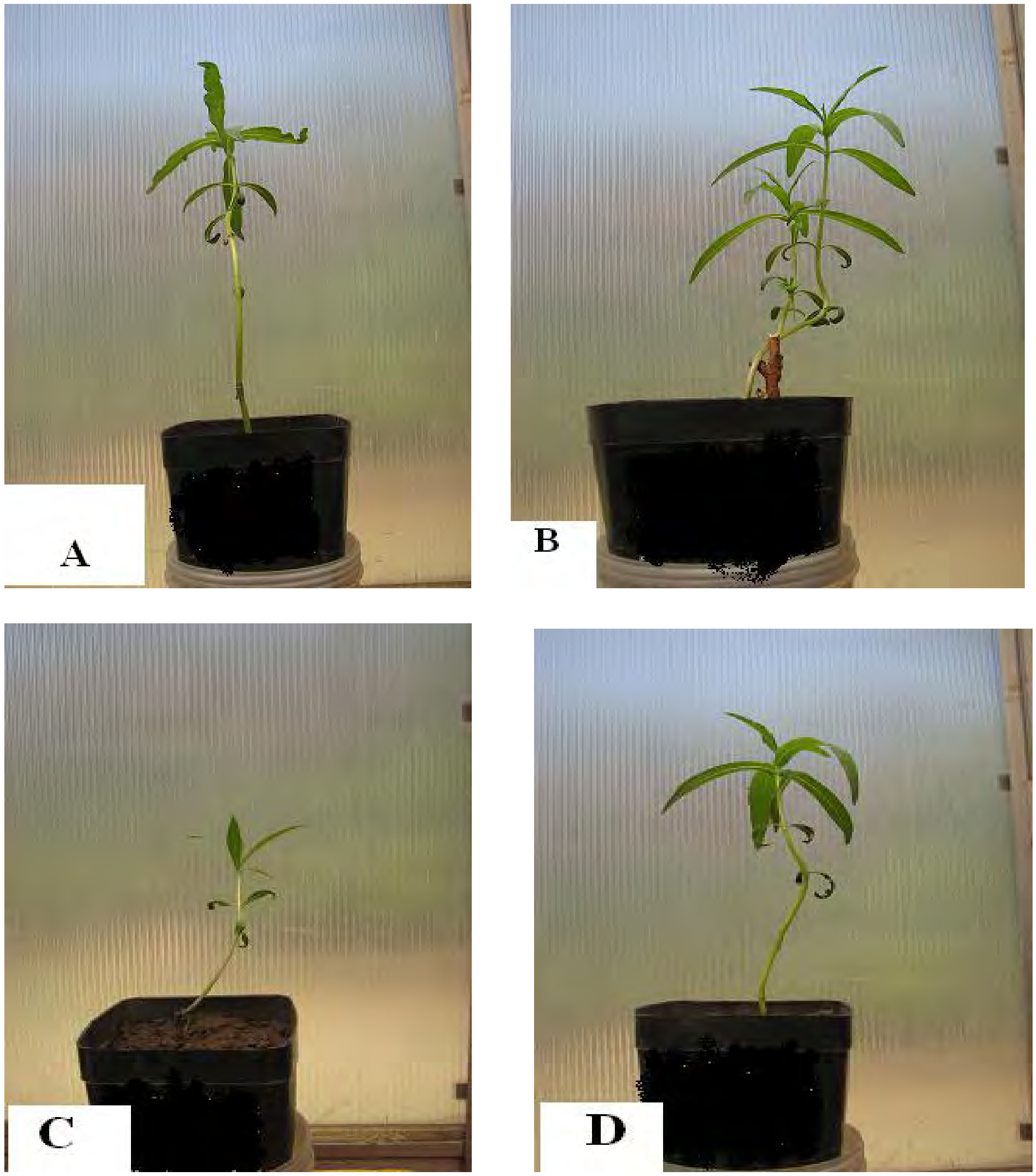



Fig 11. Regenerants of niger in the glasshouse. (A&B) Regenerants of Shambu from anthers induced callus on NN medium supplemented with 2 mg/l 2,4-D + 0.3 mg/l KN, regenerated on MS – regeneration medium supplemented with 2 mg/l KN + 1 mg/l IAA and MS- rooting medium supplemented with 0.5 mg/l IBA. (C&D) Regenerants of Forgra induced callus on $B_5$ medium supplemented with 2 mg/l 2,4-D + 0.3 mg/l KN and regenerated shoot and root on the same media compositions of the regenerants of the above variety ( Shambu). Plantlets were grown under glasshouse conditions (at a temperature of $25^0$C ± $2^0$ C and under natural light).

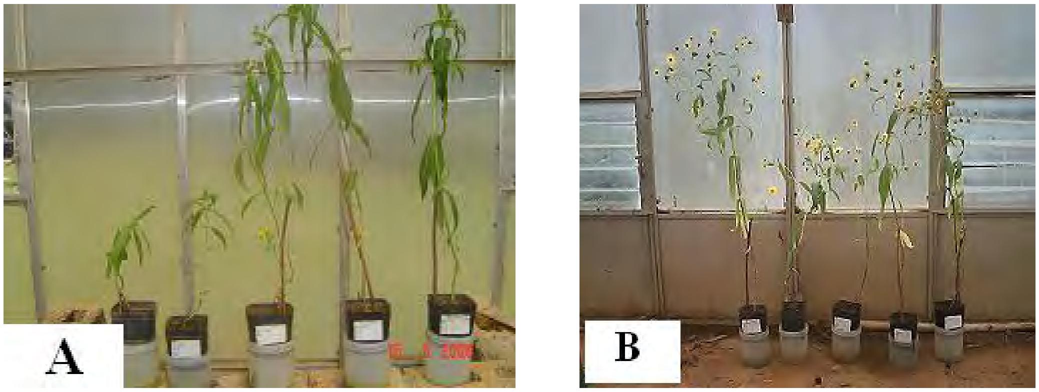



Fig 12. Anther-derived regenerants of niger at different time of growth (A) Growth status of regenerants of niger after two month of transferring into pots. (B) Growth status of regenerants of niger after three months of transferring into pots. Plantlets were grown glasshouse conditions (at a temperature of $25^0$C ± $2^0$ C and under natural light).

# 6. DISCUSSIONS

## 6.1 Determination of the appropriate harvesting stage of capitula

Selection of the appropriate harvesting stage of capitula, where the maximum number of responsive anthers can be isolated is the most critical step in anther culture. The external staging system using the extent of opening of sepal and petal emergency were used as tool in staging of capitula as Stage 1, Stage 2, Stage 3 and Stage 4. Although this kind of staging system is influenced by environmental and growth condition of donor plants, it will minimize the chance of selecting capitula with non-responsive anthers and thus facilitate to capture the responsive anthers. Based on this study, florets that are found at medium and outer side positions of Stage 1 capitula and those that are found at the center and medium position of Stage 2 capitula were determined as the best florets with maximum number of responsive anthers (Fig 3). The colour of responsive anther was found to be whitish greenish or greenish yellow. In Indian niger Sarvesh *et al*. *(*1993b) also used capitula containing whitish green anthers in anther culture of Indian niger.

## 6.2 Effect of cold-pretreatment on callus induction

In many species, responsive anthrer culture requires some kind of stress treatment of anther before culture (Kiviharju and Pehu, 1998). Often cold pretreatment at $4^0$C have been used in anther culture of many plant species to promote androgenesis (Yamagunchi *et al*., 1990). However, the time duration of cold pretreatment have to be optimized for each plant species. In this study, capitula of Stage 1 and Stage 2 were investigated for cold -pretreatment at $4^0$C. Each stage was pretreated for various hours (12, 24, 36 and 48 hours) including control (without pretreatment). According to results of this experiment, cold -pretreatment at 24 hours was found to be the best duration time to promote callus induction since capitula pretreated by this time of duration gave highest percentage of callus induction as compared to the remaining cold-pretreatment time durations. This result was consistent with the cold-pretreatment time duration applied in anther culture of Indian genotypes (Sarvesh *et al*., 1993b).

### 6.3 Effect of genotypes and culture media on callus induction

Callus induction is an essential step in tissue culture including anther culture since regeneration of plants can be obtained either through direct embryogenesis or indirectly through a callus phase. Direct embryogenesis is common in Cruciferae (mustard) and Solanaceae (tobacco) families (Charne and Beversdore, 1998). Indirect androgenesis is often occurred in cereals such as wheat and rice (Datta, 1998; Rainia, 1997). According to this study, anther-derived regenerants of niger were obtained through indirect androgenesis unlike *Brassica spp.*, which is another oil bearing crop species.

Callus induction in anther culture of many crops including niger is known to be affected by many factors. Genotype and culture media are the most prominent. In the present study, both genotypes and culture media exhibited significant (p=0.05) effect on callus induction (Table 5). Genotypes seems to be the major determinants of callus induction in anther culture of niger since wide range of variations were observed between the two varieties for callus induction percentage (7.7-29.4% for Treatment 1) and (6.4-24.8% for Treatment 2) in callus induction experiment (Table 4). However, the effect of genotype is not dependent on media or vice versa. Based on the average callus induction percentage, it was possible to select the best responsive and appropriate media for callus induction. Varieties Shambu and Fogera were the best responsive varieties and MS and $B_5$ media were found to be the best media for callus induction (Table 4). In case of media, result of this study was different from previous anther culture investigation of Indian genotypes by Sarvesh *et al* (1993b). The author found that LS medium was the best callus induction media in one of Indian genotype, Ootacamund.

### 6.4 Effect of different concentrations and combinations of PGRs for callus induction

For successful response of anther culture, it is desirable to have the appropriate combinations and concentrations of PGRs for callus induction. This study investigated 10 different combinations of 2,4-D alone or in combination with kinetin in anther culture of Shambu and Fogera. In both varieties, using 2,4-D alone at low concentration (0.5 mg/l) failed to induce callus. In his study of anther culture of Indian genotypes, Sarvesh *et al.* (1993b) also obtained similar results that niger genotype (Ootacamund) was not responsive when 2,4-D used alone at 1mg/l and 2mg/l concentration. When 2,4-D, however, combined with KN, anthers of both varieties induced calli. This indicated that kinetin is essential for triggering cell division. Although the combination of 2,4-D with KN gave better callus induction, there were considerable variations among the different combinations of these PGRs. The highest callus induction percentage was obtained at 2 mg/l 2,4-D + 0.3 mg/l KN, which was 86.7% and 73.3% for Shambu and Fogera respectively and such PGR combination can be considered as the best combination and concentration for callus induction.

### 6.5 Types of calli induced from anther culture of niger

Callus both from somatic tissue or anther can be either embryogenic or non-embryogenic depending on the explants, media and plant growth regulators (Rashid and Quraishi, 1989). According to previous studies of anther culture, usually embryogenic calli are identified by globular shaped embryo with compact morphology whereas non-embryogenic callus are usually distinguished by their large and amorphous morphology (Liang and Tang, 1987). In this study, embryogenic and non-embryogenic calli were observed in anther culture of Shambu and Fogera depends on the type of media used. MS media supplemented with 2 mg/l NAA + 0.3 mg/l KN dominantly produced non-embryogenic callus which was identified by its friable texture and large amorphous morphology. NN and $B_5$ media supplemented with the same plant growth regulator combination produced embryogenic callus that varied in its external appearance between the two media.  Embryogenic callus with shiny and smooth texture with whitish green colour was observed in NN medium while compact and rough texture with reddish-yellow colour embryogenic callus was recognized in $B_5$ medium.

### 6.6 Effect of culture media on shoot regeneration

Types of calli (embryogenic or non-embryogenic) transferred on regeneration medium and the sources of calli determine plant regeneration (Rashid and Qurashi, 1989). According to Nabors *et a*l. (1983), non-embryogenic calli are not regenerable. In this study, the NE calli also failed to regenerate when transferred to the regeneration MS medium supplemented with 1 mg/l BAP and 0.2 mg/l NAA. On the other hand, when embryogenic calli were able to regenerate but not all embryogenic calli produced complete shoots and the regeneration potential varied among source of calli. In the present study, most of the shoots regenerated were from embryogenic calli of NN and $B_5$ rather than from embryogenic calli of MS and calli from NN medium were more responsive as compared to B5.

### 6.7 Effect of different concentrations and combinations of PGRs for shoot regeneration

In the previous shoot regeneration experiment, 6- Benzylaminopurine (BAP) in combination with NAA was used. This combination, however, took more than five weeks to produce shoots. In some cases also, regenerated shoots became black and dormant when calli were transferred to MS regeneration medium supplemented with the above PGRs. It was for this reasons that investigations of the PGRs using BAP and NAA was replaced by kinetin and IAA combination. According to Mackinnon *et al*. (1987), the use of the appropriate combination of KN with IAA in MS regeneration medium promote callus differentiation and regeneration during anther culture of bread wheat and sorghum. In this study, nine different combinations of KN alone or in combination with IAA were tested to determine the best PGR combination and concentration for shoot regeneration. Among the nine combinations, the intermediate level of concentration (2 mg/l KN) with

IAA (0.5 and 1.0 mg/l) showed well-developed shoots and 2 mg/l KN + 1.0 mg/l IAA was considered as the best PGR combination for shoot regeneration in anther culture of niger particularly for the variety Shambu.

### 6.8 Rooting of shoots

In many *in vitro* cultures, rooting can be achieved with low concentration of auxin (Nitzche and Wenzel, 1977). In the present experiment of rooting of shoots, among the three different concentrations of IBA (0.5, 1 and 2 mg/l) including control, 0.5mg/l IBA was found to be the best for rooting of plantlets and further growth of shoots. According to Sarvesh *et al*. (1993b), however, MS media without PGR was found to be the best rooting media of Indian niger genotypes.

### 6.9 Growth condition of regenerants

Care should be taken in handling of regenerants since *in vitro* environment is entirely different from ambient environmental condition. Of 27 green plantlets that were transferred into pots, 55.6% of plantlets survived and the remaining 44.4% died in the growth room during acclimatization. Out of the survived 15 regenerants, only eight (53.3%) of plantlets were successfully grown in the glasshouse. The continuous interruption of electricity, poor maintenance and high temperature due to uncontrolled conditions in glasshouse might be the main reasons for the death of regenerants. Thus, keeping the optimum conditions in the growthroom and glasshouse may increase the rate of survival of plantlets.

# 7. CONCLUSIONS AND RECOMMENDATIONS

## 7.1 Conclusions

Anther culture technique enables to produce haploids or double haploids which are useful in niger improvement. Double haploids of niger improve selection efficiency of desirable traits by incorporating them in recurrent selection schemes or used in development of synthetic cultivars. Since this anther culture study might be pioneer for Ethiopian niger, this study first tried to determine the appropriate harvesting stage of capitula (buds) and time duration of cold-pretreatment of capitula (buds). Results of theses studies revealed that capitula fully or slightly covered by sepal with florets containing whitish- greenish and greenish-yellow anthers were stages with maximum number of responsive anthers. Cold pretreatment at $4^0$C is recommended for 24 hours. Callus induction experiment was conducted in order to select the best responsive varieties and appropriate media for callus induction. According to this experiment, anthers of Shambu and Fogera became the best varieties in callus induction while MS and $B_5$ were found to be the appropriate media for callus induction. In this study, it was also possible to distinguish the types of calli produced (embryogenic and non-embryogenic) and shoot regeneration potential was assessed for calli induced from different basal media. Embryogenic calli of NN followed by $B_5$ exhibited high shoot regeneration potential while embryogenic calli of MS showed the least shoot regeneration potential.

In this investigation, it was also observed that both genotypes and media significantly affected callus induction. However, the effects of the two factors were independent. Plant regeneration is significantly ($p = 0.05$) affected by callus induction media. Media affects callus induction and plant regenerations not only due to their components (kind of salts, concentration of sucrose and agar) but also the type of PGRs added to the media. Based on this fact, in this study, effect of different combinations and concentrations of PGRs were investigated and it was found that 2 mg/l 2,4-D + 0.3 mg/l KN and 2 mg/l KN + 1 mg/l IAA were the best combination and concentration plant growth regulators for callus induction and shoot regeneration respectively. It was also found that 0.5 mg/l IBA was determined as the best PGR concentration for rooting. The survival rate of Shambu regenerants seems to be higher than that of Fogera since more plantlets of Shambu survived either in the cultureroom or in the glasshouse.

### 7.2 Recommendations

Many factors are involved in anther culture of fertile green plants. This study, however, considers only the effects of genotypes, media and plant growth regulators in callus induction and regeneration. Thus, further study is necessary to include other factors such as growth condition of donor plant, stage of pollen development and incubation temperature regime.

*In vitro* plant regeneration mainly depends on embryogenic calli since these calli possess higher regeneration potential than non-embryogenic calli. Even if this study identified both embryogenic and non-embryogenic calli, the numbers of regenerants were limited in numbers. So, it is also important to investigate how to develop high frequency embryogenic calli and how to maintain these calli for long period or time.

During the present anther culture studies of niger, it was possible to find regenerated plants from anthers.  Besides this, cytological study is needed to determine the ploidy level of the regenerants. This study also used to identify whether regenerants are obtained from somatic tissue of the anther or its immature pollen. After investigating the ploidy level, it is possible to carry out dihyplodization study by treating haploids using colchicines.

Anther cultivability is a quantitative trait controlled by nuclear-encoded genes. By applying of genetics, it important to study the heritability of traits such as culture induction and plant regeneration for those best responsive genotypes. In addition to this, such characters can be detected at the molecular level by using the current biotechnological tools namely marker assisted selection (MAS) or DNA markers.

Regenerated plants survived either in the growth room or glass house were very limited so that growth or glass house facilities for controlled environment is very important.

## 8. REFERENCES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**APPENDICES**

**Appendix I**

**Table 1. A Workable external staging system of capitulea of the variety Shambu before opening of flowers**

| **Capitulea stages** | **Stage No** | **Capitulea diameter (mm)** | **State of hermaphroditic disc floret at different positions** *Floret Color* | | | *Anther Color* | | | Remark |
|---|---|---|---|---|---|---|---|---|---|
| | | | **Center** | **Medium** | **Outer** | **Center** | **Medium** | **Outer** | |
| *Capitulea fully covered by sepal* | 1 | 0.5mm-0.6mm | Whitish green | Whitish green with slight yellow colour | Greenish yellow | Whitish sticky anthers | Whitish green | Greenish yellow | Almost all anthers at any position do not release pollen |
| *Capitulea covered with slightly opened sepal and no petal emerged* | 2 | 0.5mm-0.7mm | Greenish yellow | Whitish yellow colour with green tip | Yellow colour with green tip | Greenish yellow | Whitish yellow | Yellow | The majority of anthers at the outer position release pollen |
| *Capitulea covered with opened sepal and greenish yellow petal emerged with sepal* | 3 | 0.7mm-0.8mm | Whitish yellow colour with green tip | Yellow colour with green tip | Bright yellow colour with green tip | Whitish yellow | Yellow | Bright yellow | Almost all disc florets at the outer position and the majority of them at the medium position release pollen |
| *Capitulea covered with opened sepal and yellow petal emerged with sepal* | 4 | 0.8mm-0.9mm | Yellow colour with green tip | Bright yellow colour with green tip | Deep yellow | Yellow | Bright yellow | Deep yellow | Almost all anthers in disc florets at the outer, medium and the majority at center release pollen. |

**Table 2. Effect of pre-treatment on callus induction of anthers taken from the variety Shambu**

| Pretreatment duration (hours) | No of anthers cultured | Capitulea stage | No of anthers produce calli (3-4 weeks after culture) | Callus induction (%) |
|---|---|---|---|---|
| | | ***Stage 1 capitulea*** | | |
| **0** | 30 | | 3 | 10 |
| **12** | 30 | | 16 | 53.3 |
| **24** | 30 | | 21 | 70 |
| **36** | 30 | | 18 | 60 |
| **48** | 30 | | 12 | 40 |
| | | ***Stage 2 capitulea*** | | |
| **0** | 30 | | 5 | 13.3 |
| **12** | 30 | | 17 | 56.7 |
| **24** | 30 | | 23 | 76.7 |
| **36** | 30 | | 18 | 60 |
| **48** | 30 | | 13 | 43.3 |

*Key:* $1^{st}$ stage = Capitulea fully covered by sepal
$2^{nd}$stage = Capitilea covered with slightly opened sepal and no petal emerged
Note: Callus induction medium= MS + Hormones (2.0mg/l 2,4-D and + 0.3 KN) ,Cold Pre-treatment **at $4^0$c**

**Table 3a.Callus induction percentage on anther culture of four niger varieties using MS media supplemented with 2, 4-D + KN and NAA+KN.**

| Varieties | PGRs (mg/l) | No of anthers cultured | No of anthers induced callus (3-4 weeks after culture) | Callus induction rate (%) |
|---|---|---|---|---|
| **Shambu** | 2,4-D: 2 + 0.3 KN | 38 | 14 | 36.8 |
| | NAA: 2 + 0.3 KN | 40 | 12 | 30.0 |
| **Kuyu** | 2,4-D: 2 + 0.3 KN | 39 | 5 | 12.8 |
| | NAA: 2 + 0.3 KN | 40 | 7 | 17.5 |
| **Esete** | 2,4-D: 2 + 0.3 KN | 39 | 6 | 15.4 |
| | NAA: 2 + 0.3 KN | 38 | 5 | 13.2 |
| **Fogera** | 2,4-D: 2 + 0.3 KN | 38 | 11 | 28.9 |
| | NAA: 2 + 0.3 KN | 40 | 11 | 27.5 |

**Table 3b. Callus induction percentage on anther culture of four niger varieties using $B_5$ media supplemented with 2, 4-D and NAA.**

| Varieties | PGRs (mg/l) | No of anthers cultured | No of anthers induced callus (3-4 weeks after culture) | Callus induction rate (%) |
|---|---|---|---|---|
| **Shambu** | 2,4-D: 2 + 0.3 KN | 38 | 16 | 42.1 |
| | NAA: 2 + 0.3 KN | 40 | 14 | 35.0 |
| **Kuyu** | 2,4-D: 2 + 0.3 KN | 39 | 2 | 5.1 |
| | NAA: 2 + 0.3 KN | 40 | 3 | 12.5 |
| **Esete** | 2,4-D: 2 + 0.3 KN | 40 | 3 | 7.5 |
| | NAA: 2 + 0.3 KN | 40 | 1 | 2.5 |
| **Fogera** | 2,4-D: 2 + 0.3 KN | 38 | 13 | 34.2 |
| | NAA: 2 + 0.3 KN | 39 | 13 | 33.3 |

**Table 3c. Callus induction percentage on anther culture of four niger varieties using $N_6$ media supplemented with 2, 4-D and NAA.**

| Varieties | PGRs (mg/l) | No of anthers cultured | No of anthers induced callus (3-4 weeks after culture) | Callus induction rate (%) |
|---|---|---|---|---|
| **Shambu** | 2,4-D: 2 + 0.3 KN | 38 | 9 | 23.7 |
| | NAA: 2 + 0.3 KN | 38 | 8 | 21.1 |
| **Kuyu** | 2,4-D: 2 + 0.3 KN | 38 | 3 | 7.9 |
| | NAA: 2 + 0.3 KN | 39 | 3 | 7.7 |
| **Esete** | 2,4-D: 2 + 0.3 KN | 39 | 1 | 2.6 |
| | NAA: 2 + 0.3 KN | 40 | 1 | 2.5 |
| **Fogera** | 2,4-D: 2 + 0.3 KN | 40 | 5 | 12.5 |
| | NAA: 2 + 0.3 KN | 40 | 4 | 10.0 |

**Table 3d. Callus induction percentage on anther culture of four niger varieties using LS media supplemented with 2, 4-D and NAA.**

| Varieties | PGRs (mg/l) | No of anthers cultured | No of anthers induced callus (3-4 weeks after culture) | Callus induction rate (%) |
|---|---|---|---|---|
| **Shambu** | 2,4-D: 2 + 0.3 KN | 40 | 6 | 15.0 |
| | NAA: 2 + 0.3 KN | 38 | 5 | 13.2 |
| **Kuyu** | 2,4-D: 2 + 0.3 KN | 40 | 2 | 5.0 |
| | NAA: 2 + 0.3 KN | 38 | 1 | 2.6 |
| **Esete** | 2,4-D: 2 + 0.3 KN | 38 | 2 | 5.3 |
| | NAA: 2 + 0.3 KN | 40 | 3 | 7.5 |
| **Fogera** | 2,4-D: 2 + 0.3 KN | 40 | 4 | 10.0 |
| | NAA: 2 + 0.3 KN | 40 | 3 | 7.5 |

**Table 4a. Callus induction percentage under different combinations and concentrations of 2,4-D and KN in anther culture of the variety Shambu.**

| Treatments | PGRs (mg/l) | No of anthers cultured | No of anthers responded | Callus induction (%) (3-4 weeks after culture) |
|---|---|---|---|---|
| **A** | 0.5 2,4-D | 30 | 0 | 0 |
| **B** | 1.0 2,4-D + 0.1 KN | 30 | 4 | 13.3 |
| **C** | 1.0 2,4-D + 0.3 KN | 30 | 7 | 23.3 |
| **D** | 1.0 2,4-D + 0.5 KN | 30 | 5 | 16.7 |
| **E** | 2.0 2,4-D + 0.1 KN | 30 | 15 | 50.0 |
| **F** | 2.0 2,4-D + 0.3 KN | 30 | 26 | 86.7 |
| **G** | 2.0 2,4-D + 0.5 KN | 30 | 18 | 60.0 |
| **H** | 3.0 2,4-D + 0.1 KN | 30 | 12 | 40.0 |
| **I** | 3.0 2,4-D + 0.3 KN | 30 | 10 | 33.3 |
| **J** | 3.0 2,4-D + 0.5 KN | 30 | 7 | 23.3 |

**Table 4b. Callus induction percentage under different combinations and concentrations of 2,4-D and KN in anther culture of the variety Fogera.**

| Treatments | PGRs (mg/l) | No of anthers inoculated | No of anthers responded | Callus induction (%) (3-4 weeks after culture) |
|---|---|---|---|---|
| **A** | 0.5 2,4-D | 30 | 0 | 0 |
| **B** | 1.0 2,4-D + 0.1 KN | 30 | 3 | 10 |
| **C** | 1.0 2,4-D + 0.3 KN | 30 | 5 | 16.7 |
| **D** | 1.0 2,4-D + 0.5 KN | 30 | 4 | 13.3 |
| **E** | 2.0 2,4-D + 0.1 KN | 30 | 13 | 43.3 |
| **F** | 2.0 2,4-D + 0.3 KN | 30 | 22 | 73.3 |
| **G** | 2.0 2,4-D + 0.5 KN | 30 | 16 | 53.3 |
| **H** | 3.0 2,4-D + 0.1 KN | 30 | 8 | 26.7 |
| **I** | 3.0 2,4-D + 0.3 KN | 30 | 6 | 20.0 |
| **J** | 3.0 2,4-D + 0.5 KN | 30 | 4 | 13.3 |

**Table 5. Shoot regeneration percentage of anthers induced in three culture media in anther culture of Shambu and Fogera.**

| Variety | Callus induction media | No of calli transferred | No of calli showing shoot | Percentage of shoot regeneration |
|---|---|---|---|---|
| **Shambu** | MS | 18 | 6 | 33.3 |
| | $B_5$ | 18 | 12 | 66.7 |
| | NN | 18 | 16 | 88.9 |
| **Fogera** | MS | 18 | 6 | 33.3 |
| | $B_5$ | 18 | 10 | 55.6 |
| | NN | 18 | 14 | 77.8 |
| | | Total= 108 | Total= 64 | Mean = 33.3 |

**Table 6. Percentage of shoot regeneration of calli in anther culture of the variety Shambu**

| Treatments | PGRs (mg/l) | No of calli inoculated | No of cultures with shoots | Percentage of cultures with shoots |
|---|---|---|---|---|
| **A** | 1.0 KN | 12 | 0 | 0 |
| **B** | 1.0 KN + 0.5 IAA | 12 | 1 | 8.3 |
| **C** | 1.0 KN + 1.0 IAA | 12 | 3 | 25 |
| **D** | 2.0 KN | 12 | 2 | 16.7 |
| **E** | 2.0 KN + 0.5 IAA | 12 | 5 | 41.7 |
| **F** | 2.0 KN + 1.0 IAA | 12 | 10 | 83.3 |
| **G** | 2.0 KN | 12 | 3 | 25 |
| **H** | 3.0 KN + 0.5 IAA | 12 | 2 | 16.7 |
| **I** | 3.0 KN + 1.0 IAA | 12 | 2 | 16.7 |
| | | Total =108 | Total = 28 | Mean = 25.9 |

**Table 7. Number roots emerged during culturing of regenerated shoots of Shambu and Fogera in MS rooting media supplemented with different concentration of IBA.**

| Variety | Replicates | Concentration of IBA (mg/l) | | | |
|---|---|---|---|---|---|
| | | Control | 0.5 | 1.0 | 2.0 |
| | | *Treatment 1* | | | |
| | 1 | 8 | 13 | 9 | 3 |
| Shambu | 2 | 9 | 12 | 11 | 2 |
| | 3 | 10 | 9 | 8 | 5 |
| | 4 | 7 | 10 | 10 | 4 |
| | Mean | **8.5** | **11.0** | **9.5** | **3.5** |
| | | *Treatment 2* | | | |
| | 1 | 6 | 8 | 0 | 0 |
| Fogera | 2 | 8 | 7 | 8 | 0 |
| | 3 | 7 | 9 | 7 | 2 |
| | 4 | 9 | 10 | 0 | 0 |
| | Mean | **7.5** | **8.5** | **7.5** | **2.0** |

**Appendix II**

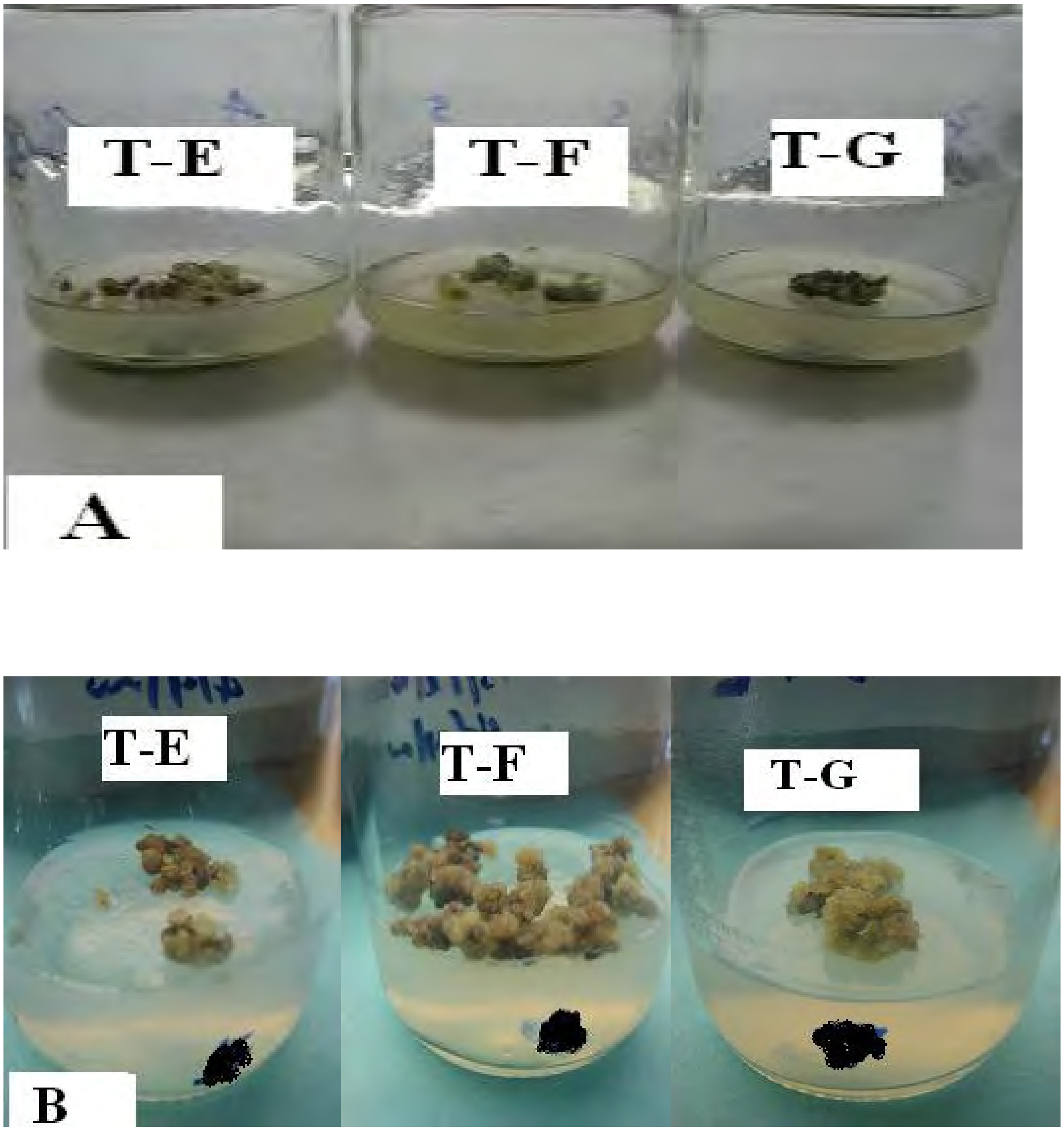


Fig 1. Induced calli of niger at different levels of 2,4-D and KN.

T-E, T- F and T-G= refers to treatments labeled in the data

(A) Calli produced in anther culture of Shambu in MS media supplemented with (2 mg/l 2, 4-D + 0.1mg/l) (T-E), (2 mg/l 2, 4-D + 0.3 mg/l) (T-F) and (2 mg/l 2, 4-D + 0.5 mg/l) (T-G)

(B) Calli produced in anther culture of Fogera in MS media supplemented with (2mg/l 2, 4-D + 0.1 mg/l) (T-E), (2 mg/l 2, 4-D + 0.3 mg/l) (T-F) and (2 mg/l 2, 4-D + 0.5 mg/l) (T-G)

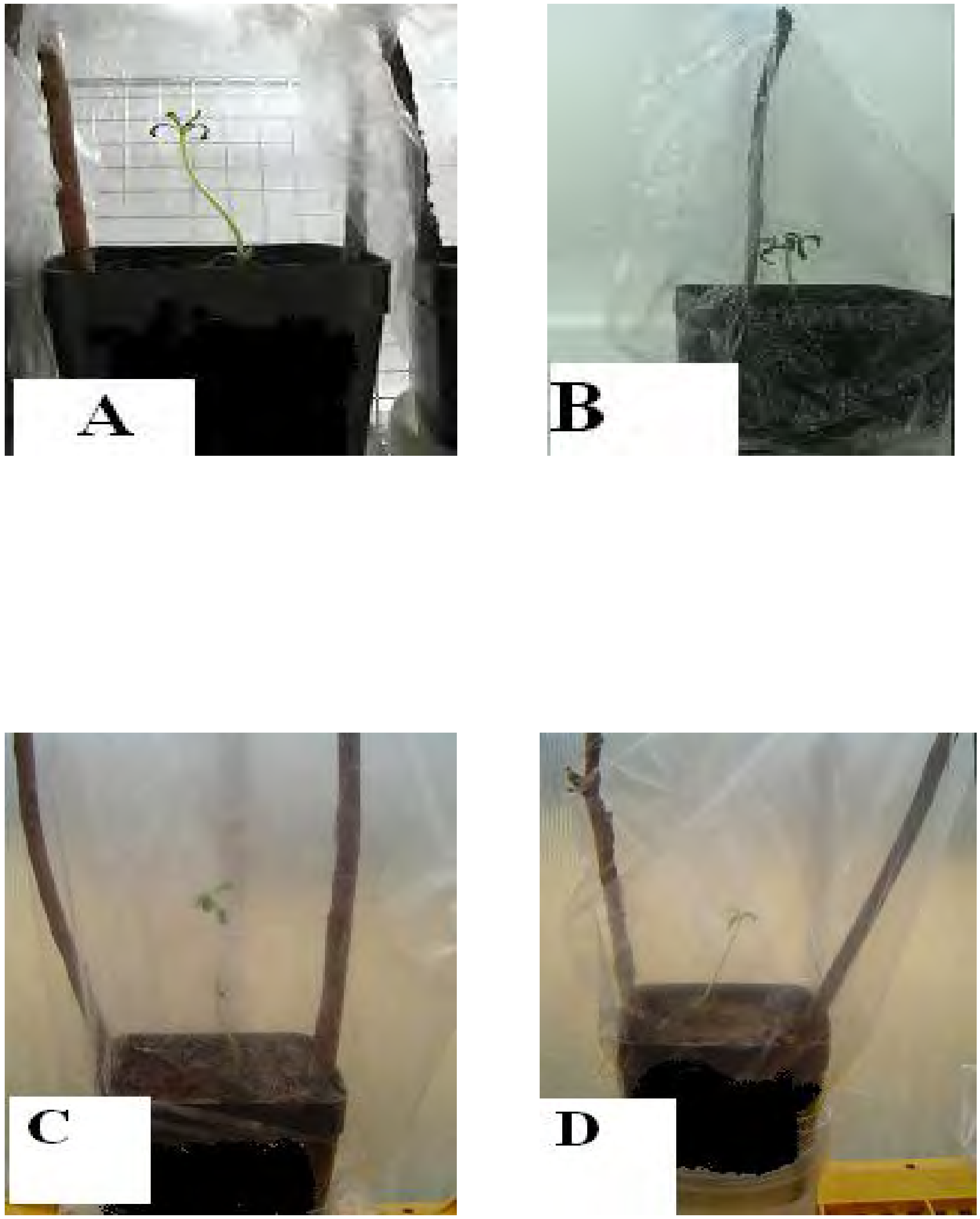


Fig 2. Some of regenerants of niger died in growthroom and glasshouse. (A) and (B) Regenerants of Fogera and Shambu died in the growthroom, respectively. (C) and (D) Regenerants of Shambu wilted in glasshouse.