
ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOLOGY


# PLANT REGENERATION FROM ANTHER CULTURE OF FOUR VARIETIES OF ETHIOPIAN MUSTARD (*BRASSICA CARINATA* A.BRAUN)


BY

YEMISRACH ZEWDU BELAY


*A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES, ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY (BOTANICAL SCIENCE)*


ADDIS ABABA, JULY, 2010

## ACKNOWLEDGEMENT

I would like to express my most sincere appreciation and gratitude to my advisor Dr. Tileye Feyissa and co-advisor Mr. Mistiru Tesfaye for their keen interest, encouragements and unreserved guidance throughout the course of the study.

I am very grateful to BahirDar University for offering me study leave and financial support to pursue my study.

My family, Gashe, Talemye, TG, Fiker, Selam and Abby, you were always there saving my blue days all the time, I know thank you is just flat. So, I love you all and I am so honored to have you.

I would also like to give many thanks to dear friends Dawit Beyene, Workia Ahmed, Mahlet Hailu, Beza Kinfe, Kebedech Limenew, Woinshet Defar, Milen Medhane and Yahya Ali, for their great support and encouragement during those times.

It is also grateful to thank the Ethiopian Institute of Agricultural Research (EIAR), Holeta Agricultural Research Center for their goodwill to let me use their laboratory facilities and transportation. To the Biotechnology staff, you all are people with good hearts, so thank you for supporting me.

I am so grateful to Dr. Eyasu Abrha for his keen moral support and materials, Dr. Baye for his constructive suggestions on aphid control, Dr. Kifle Dagne for his unreserved help in the cytogenetics and Guluma Tenkolu for his great help.

I forget to mention names of some really wonderful people, I am so grateful to all of you who helped me in any way.

**TABLE OF CONTENTS**

**Page**

# LIST OF FIGURES

**LIST OF TABLES**

Page

## ACRONYMS

$B_5$ - Gamborge medium

BAP – Benzyl amino purine

$GA_3$ – Gibberlic acid

HARC - Holetta Agricultural Research Center

IAA- Indole acetic acid

IBA – Indole butyric acid

MS - Murashige and Skoog medium

$N_6$ - Chu medium

NAA – Naphthalene acetic acid

PGR - Plant growth regulator

**ABSTRACT**

*Brassica carinata* A. Braun is an amphi-diploid species originated from interspecific hybridization between *Brassica nigra* and *Brassica oleracea* in the highlands of Ethiopia and adjoining regions of East African and Mediterranean coast. The crop has many desirable agronomic traits, but, with oil quality constraints like high erucic acid and glucosinolate contents. The application of anther culture could help ito improve the oil quality and other characteristics of the crop by developing haploid and doubled haploid lines within a shorter period of time than the conventional method. Four varieties of *Brassica carinata,* namely, Holeta 1, PGRCE 07/1/2, S-67 and Yellow Dodola, obtained from HARC and four basal media NN, $B_5$, MS and $N_6$ were used. In this study the experimental parameters like the effect of basal media, bud length and heat shock for anther derived plantlet regeneration were considered. The most responsive bud stages were flower buds with a length range of 1.5 - 2.5mm for all the varieties used. Regarding the heat treatment effects, incubating the cultures at a temperature of $32^0$C for 24h with a humidity of 70% and transferring to continuous $25^0$C dark culture condition gave the best result. The maximum embryo numbers were found to be 41.3, 36.3 and 26.0 for varieties PGRCE07/1/2, Yellow Dodolla and S-67, respectively, on $B_5$ media supplemented with 1mg/l of NAA and 0.5mg/l of BAP. Holeta 1 scored the maximum mean number of embryos (40.3) on NN medium supplemented with similar PGR concentration. Germination of embryos was carried out on $B_5$ medium supplemented with 0.02mg/l BAP and 0.02mg/l IAA and growth regulator free $B_5$ medium, and the latter was found to be the best regeneration medium for all the varieties. Acclimatization and hardening was successful with 100% survival rate for varieties PGRCE 07/1/2 and S-67, and the survival rates for Holeta 1 and Yellow Dodola were 97.3% and 96.3%, respectively.

**Key Words / phrases**: acclimatization, androgenesis, basal media, embryogenesis, plant growth regulators, plantlet.

# 1. INTRODUCTION

*Brassica carinata* A. Braun, commonly known as Ethiopian mustard is a amphi-diploid species that belong to the family Brassicaceae. It originated through interspecific hybridization between *Brassica nigra* and *Brassica oleracea* in the highlands of Ethiopia and adjoining regions of East African and Mediterranean coast (Simmonds, 1979; Choung and Bevedrof, 1985).

It is an annual vegetable growing to 1.6 m at fast rate with hermaphrodite flowers which are pollinated by bees. The plant is self pollinated with about 30 – 50 % outcrossing (Rakow, 2004). It prefers light, medium to heavy soils and grows better in well – drained moist soil (Hiruy Belayneh, 1985).

Edible oil is obtained from the seed of *Brassica carinata* which contains high erucic acid that is toxic. However, modern cultivars that contain very little erucic acid concentration or almost free of erucic acid have been produced that can be used for the production of edible oil (Velasco *et al*., 2004). Recent findings reported that among the members of the genus *Brassica*, the seed oil of *Brassica carinata* can be used to produce biodiesel and erucic acid derivatives can be used as chemical additives in plastic, tannery and cosmetic industries (Bozzini *et al*., 2007).

Traditionally, *B. carinata* seeds also called 'Gomenzer' in Amharic are used to grease clay pan used for baking traditional Ethiopian bread "enjera", to cure certain ailments or stomach upsets and to prepare some beverages. The leaves of young plants are good sources of vegetable relish (Nigussie Almayehu *et al*., 2003). In addition it is used in the farming systems, especially in large scale farms as a break crop for cultivation of cereals with comparable ecological amplitude (Oleszek, 1987). There are many advantages of using *Brassica carinata* as cover crops, such as suppressing nematodes, diseases and insects (Al-khatib and Boydston, 1999). Other studies have also shown that high glucosinolate containing *Brassica carinata* can efficiently control not only weeds but also other soil-borne pests (Brown and Morra, 1997).

*Brassica carinata* possesses so many desirable traits like high rusticity and adaptability, low chemical input requirement, strong resistance to disease and water shortages, delayed pod

shattering or low pod dehiscence even after maturation, simple mechanization, high amount of crop residue and simple insertion in cereal rotation, drought and salt tolerance, pest resistance, high yield and large seed size (Lazzeri and Avino, 2009). But its oil quality and content is the main constraint (Velasco *et al.,* 1998).

Through anther culture, by producing haploids and doubled haploids, it is possible to develop inbred lines with complete homozygousity, for the production of haploids and doubled haploids, which facilitates biotechnological approaches like genetic engineering, production of new varieties, production of inbreds for hetrosis breeding, selection of stable resistant lines against biotic and abiotic stresses and purification of male lines (Galli *et al*., 1998).In addition it is possible to apply marker assisted selection that would help to improve the crop which in turn will upgrade its economical and commercial value.

In Ethiopia, *B. carinata* breeding is only restricted to conventional breeding with repeated selfing to develop new varieties and to attain pure lines which is time consuming. Therefore the regeneration of plants from anther culture could be applied in improving the oil quality and other desirable traits of the crop by developing homozygous inbred lines in shorter time. In addition, the self compatibility and out-crossing ability makes the anther derived homozygous lines to be part of the mass and recurrent selection that improves even other *Brassica* species (Nigussie Alemayehu *et al*., 1996).

Studies proved that, there are several factors that influence *in vitro* anther and microspore culture: genotype and growth conditions of donor plants, for instance dense planting and developmental stage of microspore or pollen development affects androgenic embryo response, bud size affected embryo response in *Brassica campestris* (Vincente and Dias, 1996). Similarly, culture medium can also affect anther or microspore culture. A research on *Brassica napus* anther culture showed that embryogenic response was highly affected by sucrose concentration (Kott and Beversdorf, 1988). In addition, incubation periods or temperature treatments have been identified as critical variables in androgensis (Lashermes, 1990).To overcome the influence of the mentioned factors, refinement of experimental parameters depending on the local conditions or developing suitable protocol is necessary.

Anther and microspore culture has been made effective in most species of *Brassica* including *Brassica napus, Brassica oleracea, Brassica campestris, Brassica junceae Brassica rapa* and others (Keller and Armstrong, 1979; Lichter, 1982; Choung and Beversdorf, 1985; Huang *et al*., 1990).

In this study, assessment of plant regeneration through anther culture of *Brassica carinata* by optimizing basal medium for embrogenesis and plant regeneration, identifying appropriate bud stage for anther excision, determining suitable incubation period or temperature treatment for embryogenesis and investigating the optimum culture conditions for rooting and acclimatization of anther derived plantlets has been carried out.

# 2. LITERATURE REVIEW

## 2.1 Crop description

### 2.1.1 Morphological description

Ethiopian mustard is an erect annual, occasionally biannual or perennial crop grown as oilseeds or as a leafy vegetable. The seedling of the crop emerges with epigeal germination, whereby the cotyledons appear above the ground that enables them to be photosynthetically active to offset the negative consequences of insufficiency of the reserve food within the seed. *B. carinata* develop a substantially elongated taproot reaching up to one meter or more with numerous laterals. The stem and leaves are green or deep green with many branches and short petiole respectively.

The inflorescence of *B. carinata* is an elongated raceme, borne terminally on the main stem and branches. The flowers are typically bright-yellow although flower color varies from orange to creamish white. The fruit is a long narrow pod which is botanically a silique (pl = siliqua) consisting of two carpels separated by a false septum. The seeds are mainly embryonic and are often small with the dominant color of brown and sometimes yellow (Setia and Richa, 1989).

### 2.1.2 Taxonomy

*Brassica carinata* A. Braun belongs to family Brassicaceae (formerly known as Cruciferae); order - Capparales, genus –*Brassica* and species – *carinata* (Edwards *et al*., 2000).The name *B. carinata* was for the first time given by A. Braun in 1841. The species is known to have various scientific synonyms like *Brassica intergrifolia* Var. Carinata (West) Rupr (1860), *Melanosinapis abyssinica* Hort. ex Regel, *Sinapis abyssinica* A. Braun (1856) (Edwards *et al.,* 2000).The plant is grown as a leaf vegetable with a mild flavor. It is known as Gomenzer or *Yabesha gomen* in Amharic (Zemede Asfaw, 1995).

Named varieties in other parts of the world include Abyssinian mustard, Ethiopian mustard, Ethiopian rape seed (Europe, trade mark name), Figiri (Zambia), Loshuu (Tanzania), Sukuma wiki (Kiswahli) and in Texas United states of America as Tamu- Texsel (Schippers, 2002).

Cytotaxonomic evidences suggest that *Brassica carinata* A. Braun (n=17) is amphidiploid species evolved through natural hybridization between *B. nigra* (BB n=8) and *B. oleracea* (CC, n=9), followed by chromosome doubling in the highlands of Ethiopia and the adjoining regions of East Africa and the Mediterranean coast where both the parental species were sympatric (Simmond, 1979; Hemingway, 1995). The evolutionary relationships of the *B. carinata* and other oilseed *Brassicas* usually illustrated by U–triangle (Fig. 1), which had got this name from Japanese scientist Nicolas U (1935) who developed the relationship triangle.

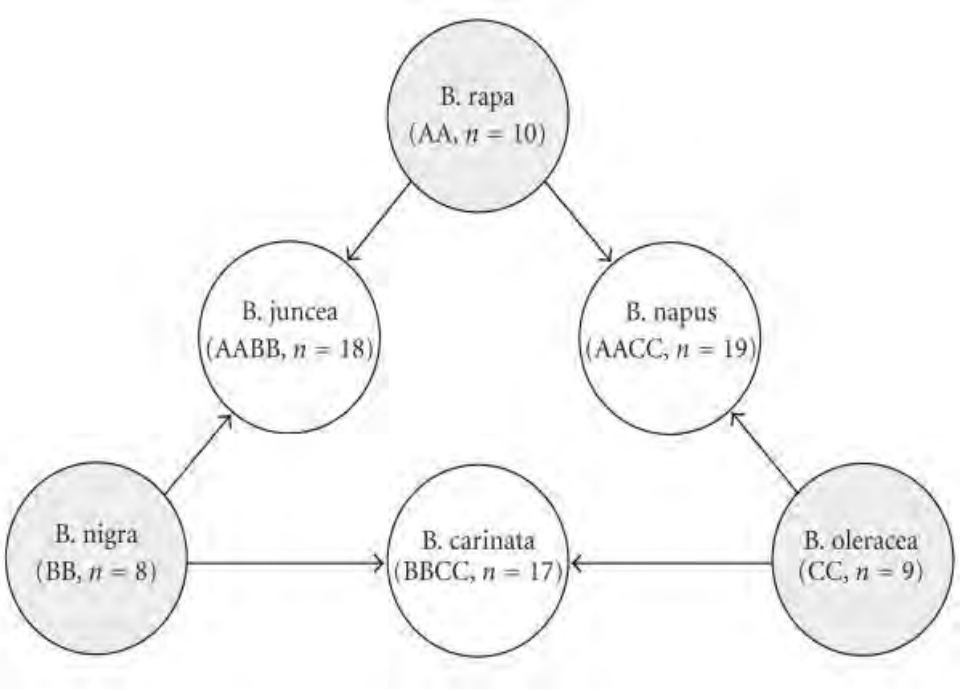


**Fig.1** Genomic relationships of the major oilseed *Brassica* spp. (after U, 1935)

### 2.1.3 Ecology, agronomy and production status

*B. carinata* grows in most part of Ethiopia at medium to high altitudes ranging from 2000 to 2600m asl, in areas of the country where rain fall and temperature amount ranges from 700–1000mm and 15-20$^{o}$C, respectively (Adefris Teklewold and Nigussie Alemayehu, 1996). The crop is often grown as a backyard crop on humus-rich soils by small holder farmers. In some potential areas where Ethiopian mustard is grown in large scale, the crop prefers moderately heavy and well drained soils with pH of 6.5- 7.6 (Nigussie Alemayehu *et al*., 1996).

The two most important oil seed *Brassica* species known in Ethiopia are *B. carinata* and *B. napus.* The latter is introduced as an early maturing species, more determinate with reduced branches and suitable for mechanization. Ethiopian mustard, on the other hand, is long maturing species that gives an advantage of higher yield due to its long growing season in the areas where rain fall amount and distribution is optimum (Hiruye Belayneh, 1985). Field research in the central highlands of Ethiopia confirmed that higher yields for oil seed *Brassica* can be obtained when the crop is planted during the on-set of the main rainy season (Hiruy Belayneh and Nigussie Almayehu, 1986). For most localities, this time is expected to occur between late May and mid-June. According to the agronomic studies by Hiruy Belayneh and Nigussie Almayehu (1986), a seed rate of 10-15 kg/ha with a fertilizer rate of 46/69 Kg/ha of $N/P_2O_5$ is optimum depending on the season and planting techniques. Under normal conditions *Brassica carinata* has a vigorous growth even on marginal soils, the vegetation height reaches 1.60-1.70 m; the stem and leaves are deep green, with anthocyanin in cold periods; leaves are waxy with seeds high in the erucic acid and glucosinolates. Therefore, not commonly used for production of edible oil (Rakow, 2004).

The major Ethiopian mustard growing areas are located in Gojam, Arsi, Bale, Eastern Wollega, Central and Southern Shewa. The total production hectarage of the crop during the main growing as well as off-seasons was steadily increasing from 26,020 ha in 2003/04 to above 34,580 ha in 2007/08 (CSA, 2008/09). Correspondingly, total production has increased from 29,284 tons to 47,721 tons during the same periods. *Brassica carinata* has become an object of interest in Canada (Rakow, 1995), Spain (Velasco *et al*., 1995), Italy (Bozzini *et al*., 2007) and India (Singh, 2003) due to its draught tolerance superiority over the other oilseed *Brassicas*.

*B. carinata* is found exclusively in Ethiopia, but recently it has been cultivated in different parts of the world. Including Southern Africa (Zambia), West Africa (Sera Leon and Guinea) and in Asia (India, China, Bangladeshi and Indonesia) as a vegetable crop along with other members of the genus. This crop is also extensively cultivated in Eastern Europe and U.S.A. as animal and fish fodder (FAO, 2007).

## 2.1 4 *Brassica carinata* as a source of important agronomic traits

Because of its good agronomic characters, the biotechnological applications are carried at different research institutions and countries like Canada. Breeding efforts have focused on the development of improved *Brassica carinata* with increased seed oil content and modified fatty acid profiles, as well as improved agronomic characters including early maturing forms (Yitbarek Semeane, 1992).

Ethiopian mustard is known for its agronomic qualities which are rare or absent in other oilseed *Brassica* species. These include relatively large seed size (Getinet Alemaw *et al.,* 1996), high heat and drought tolerance, good resistance to blackleg disease and reduced amount of seed shattering problem (Gugel *et al.,* 1990). It is also resistant to insect pests like aphids and flea beetles and some accessions have high levels of resistance to alternaria black spot. Moreover, Ethiopian mustard is a promising oilseed crop for semi-arid areas where it has better agronomic performance than its close relative *B. napus* L. (Deharo *et al.,* 1998).

Weed-suppressive effects of *Brassica carinata* have been noted in numerous reports (Oleszek, 1987; Vera *et al.,* 1987; Krishnan *et al.,* 1998). Al-khatib and Bkoydston (1999) reported that there are many advantages to using *Brassica* as cover crops, such as suppressing nematodes, diseases and insects. Other studies have also shown that high glucosinolate-containing *Brassica* species can efficiently control not only weeds but also other soil-borne pests (Brown and Morra, 1997). Furthermore, *B. carinata* is noted to be highly resistant to blackleg, *Lepitosphaeria maculans* (Gugel *et al.,* 1990), white rust, *Albugo candida* (Singh and Singh, 1988), and *Sclerotinia* and *Phyllotreta cruciferae* (Askew, 2004).

*Brassica carinata* pods are more resistant to shattering than rape (*Brassica napus*), providing more time, easier complete mechanical harvesting and variability in seed oil content. In addition the species is largely self-pollinated (Bozzini *et al*., 2007). It has a relatively large seed size that contains 26 to 40 percent oil, with erucic acid making about 40 percent of the fatty acids. This makes it unsuitable for human consumption. However, the oil can be used as industrial feedstock in biodiesel production, in tannery, biopesticide production, in textile and cosmetics (Phelps, 2008).

*B. carinata* is well adapted to saline environment with better salinity tolerance than the other *Brassica* species, in a previous study with four *Brassica* species considerable inter-specific variation in salt tolerance was found. *B. carinata* and *B. napus* were found to be salt tolerant as compared with *B. juncea* and *B. campestris.* This can be exploited through selection and breeding for enhancing salt tolerance of other crops (Ashraf and Sharif, 1997).

The glucosinolate content in *Brassica carinata* is high compared to other *Brassica* species which makes the crop suitable for pharmacological approaches. Characteristic odors and flavors of *Brasicas* are due to the glucosinolate hydrolysis products (Daxenbichler *et al*., 1991). Recently more than 85 types of glucosinolates are identified and there is an interest in their pharmacological role in the prevention of disease and their use as insect attractants or repellants and their role in chemical defense against pathogens, herbivores and weeds (Angelini *et al*., 1998).

For use as leafy vegetables, the preferred traits are large leaf size, late flowering, many leaves per plant and tolerance to major diseases and pests. *B. carinata* produced the greatest number of leaves and in height clearly exceeded both parental species and others (Courtney *et al*., 2005). In the case of stem biomass, *B. carinata* was much larger than any other *Brassica* species. Among the allopolyploids, both *B. carinata* and *B. juncea* were characterized by phenotypic values higher than either of their respective parents.

Popular cultivars of *Brassica carinata* identified around Kenya including White Figiri, Purple Figiri, Lushoo, Mbeya Green and Lambo are found to be resistant to major disease like blackleg and like white rust (Muthoni, 2010).In addition to biotic stress resistance, researchers reported

that *B. carinata* is an abiotic stress tolerant plant and can be used as a source of several genes and proteins that could be applied to several crops like tomatoes, sorghum, cotton, rice, ground nuts, chick pea, sugar cane and several others using genetic engineering (Bansal, 2002).

Drought tolerance in *B. carinata* is related to the better-developed root system (Liang *et al*., 1992). Development of drought-resistant crops through selection and breeding is of considerable economic value for increasing crop production in areas with low precipitation or without any appropriate irrigation system (Ashraf and Sharif, 1998). This has dual benefit for *B. carinata* breeders. The drought resistant genotypes can be of direct use on drought-hit areas with substantial management and could be a good source of resistant genes (Moffat, 2002).

## 2.2 Economic importance of *B. carinata*

According to Grubben and Oyen (2004) *B. carinata* was categorized as one of the most important traditional East African vegetables both in the amount of crop area and human nutrition together with *Brassica juncea*, *Brassica napus* and *Brassica oleracea.* Unlike the nutritive value of white cabbage the nutrient composition of these green leaf cabbages is high. *B. carinata* cultivar TexSel greens, grown in northeast Africa are reported to be higher in protein than spinach (Warwick *et al*., 2006). Reports of Ethiopian kale seed protein analyses have reveal high content of amino acids like glutamic acid, arginine and proline (Malik, 1990).

Ethiopian mustard is traditionally used for many purposes, such as greasing traditional bread-making clay pan, curing certain ailments and preparing beverages (Nigussie Alemayehu *et al*., 2001). The oil is also used for cooking in the country, usually adulterated with oils from niger seed or linseed. Besides the utilization of the crop as oil source, Ethiopian mustard is commonly used as a vegetable in Ethiopia. The young leaves and branches are usually used as a side dish after it has been cooked and served with the known local food of Ethiopia, 'Enjera'. In some southern part of Ethiopia, the leaf is cooked or boiled with meat and served as a main dish to be eaten with the local bread made from 'Enset' (*Ensete ventricusum*).

The crop has several industrial uses; according to (Gonzalez-García *et al*., 2009) bioethanol can be synthesized from *Brassica carinata* oil using KOH (Potassium hydroxide) as catalyst. In addition the fatty acid ethyl esters (biodiesel) from *Brassica carinata* oil were very stable or did not demonstrate rapid increase in peroxide value, acid value, and viscosity with increasing storage time. *B. carinata* can also be used for arsenic intoxicated soil treatment as a biofumignt; the plant has the ability to tolerate arsenic toxicity by accumulating the substance in its leaves like its parents *Brassica oleracea* and *Brassica nigra* (Artus, 2002).

*Brassica carinata* could also be used in pharmaceuticals, hirudin a pharmaceutical protein commonly used as anticoagulants to prevent thrombosis has been successfully expressed and purified in seeds of *Brassica napus* and *Brassica carinata* (Miao *et al*., 2008). For the production of chemical additive, nervonic oils extracted from *Brassica carinata* can be used as chemical feedstock in a number of industrial applications including polymers and polymer blends like

Nylon, polyurethane plastics and foams, coatings and adhesives including modified epoxide resins and glues, composite materials and cosmetic formulations (Stymne, 2008).

Research in USA reported that sulforaphane, a component of mustard, has been found to inhibit the proliferation of human breast cancer cells. Oral administration of either sulforaphane or its glucosinolate precursor glucoraphanin has been shown to inhibit carcinogen-induced mammary carcinogenesis in rats (Steinmetz and Potter, 1996).Components of mustard have been shown to have antimutagenic, antidiabetic, antifungal, antimicrobial and antioxidant effects (Miao *et al*., 2008). White mustard seed has been shown to inhibit colon cancer formation when added to the diets of both normal and obese rats. *Brassica campestris* has been shown to inhibit the formation of carcinogen-induced stomach and uterine cancer in mice. According to Steinmetz and Potter (1996), in addition, cruciferous vegetables have also been shown to reduce the risk of gallbladder and urinary bladder cancer and inhibit the proliferation of lung, pancreatic and prostate cancer cells.

## 2.3 Breeding, genetics and reproduction of *B. carinata*

The pollen of *B.carinata* is heavy and sticky, can be transferred from plant to plant through physical contact between neighboring plants, by wind and insects. The flowers are very attractive to honey bees which collect both pollen and nectar. Although out-crossing rates of 30-50% have been reported in *B. carinata* (Rakow and Woods, 1987), under field conditions the fertilization of ovules usually results from self-pollination (Bozzini *et al*., 2007).

In Ethiopia, systematic studies on *B.carintata* started in the early 1970's with test of single plant selected from Awassa collections (Getinet Alemaw and Nigussie Alemayehu, 1991). The overall objective of Ethiopian mustard breeding is to develop high yielding varieties with good oil and meal quality (Getinet Alemaw *et al*., 1991). The specific objectives were to develop cultivar with low erucic acid (< 5%), low glucosinolates (< 15µ moles/g of seed) for the breeding research targeting oil and meal quality. The principal aim of the genetics and breeding research on oilseed *Brassica* is generally to contribute the efforts of increasing production and productivity. In addition by enhancing acceptability of mustard and rapeseed as a source of edible and industrial or chemical oil by farmers, traders, oil-pressing industries and the public in

general. This will maximize oil production potential of the country and income-generation capacity for farmers.

Since the *B. carinata* possesses mixed mating, the breeding methods applied for self and cross-pollinated crops can be used for its improvement. Hybridization followed by pedigree selection and recurrent selection methods are those breeding procedure usually applied for Ethiopian mustard (Getnet Alemaw *et al*., 1991). Selections obtained from the above breeding methods or those obtained from collection mission evaluated by passing through different breeding stages. The breeding stages usually start from nursery or preliminary trials up to multi-location trials namely National variety trials and variety verification trials. So far three varieties of Ethiopian mustard, namely S-67, Yellow Dodola and Holetta-1 were released and found to be under production (Table 1).

Table 1. *Brassica carinata* varieties and their agronomic performance at Holeta Agricultural Research Rnstitute

| Varieties | Year of release | Maturity date | Yield (Kg/ha) | Oil content (%) |
|---|---|---|---|---|
| S-67 | 1984 | 157 | 3030 | 40.5 |
| Yello Dodola | 1986 | 156 | 3020 | 44.1 |
| Holetta-1 | 2005 | 150 | 3030 | 39.1 |
| PGRCE 07/1/2* | - | - | - | - |

PGRCE 07/1/2*= pipeline material.
Source: Highland Oil Crops Progress Report, EIAR, Holetta Center, 2007

## 2.4 Biotechnological approaches to *B. carinata* breeding

Biotechnological approaches that help for Brassica improvement includes tissue culture, microspore culture and anther culture for haploid and doubled haploid production; somatic cell fusion, molecular markers for genetic fidelity of in vitro-grown plants. Other applications include mutagenesis, biochemical studies and transformation with respect to desirable traits engineered.

**Mutation:** the techniques have improved yield and resistance to diseases and pests in *Brassica carinata* (Henderson and Pauls, 1992). Mutagenesis has led to the development of *Brassica napus* lines with a thinner seed coat, higher oil and protein content and low fiber content as well as other developments which could be applicable in *Brassica carinata* breeding.

**Hybridization:** Successful interspecies and intergeneric crosses were reported for *Brassica carinata* by several authors. The interspecies crosses were with *Brassica juncea* as male (Sharma and Singh, 1992), with *Brassica maurorum* as male (Chrungu *et al*., 1999), with *Brassica napus* both as female (Sabharwal and Dolezel, 1993) and as male (Sacristan and Gerdemann, 1986), with *Brassica nigra* as female (Attia *et al*. , 1987): with *Brassica oleracea* as female and as male (Tonguc and Griffiths, 2004; Attia *et al*., 1987), with *Brassica rapa* as female and as male (Busso *et al*., 1987; Meng *et al*., 1998).

The successful intergeneric crosses were with *Enarthrocarpus lyratus* as male (Gundimeda *et al*., 1992), with *Erucastrum abyssinicum* as male (Rao *et al*., 1996) with *Sinapis alba* as female (Sridevi & Sarla, 1996) and with *Sinapis arvensis* as female (Momotaz *et al*., 1998).

**Transformation:** Nowadays *Brassica carinata* is used as an alternative industrial oil crop in Canada by developing efficient transformation protocol and isolating the drought resistant gene PBI (Stymne, 2008). Even wax and ester products could be improved for industrial and commercial uses. An early maturing canola-like *Brassica carinata* cultivar with zero erucic acid content was developed in Canada. The crop's heat and drought tolerance would allow its production in the hotter and drier areas. This is an indication that there is a possibility to transform *B. carinata* for edible uses like its relatives.

**Protoplast fusion**: this technology was developed for *Brassica rapa* and *Brassica carinata* which permitted the production of somatic hybrids between these cruciferous species and the formation of novel cybrids and hybrids (Beranek *et al*., 2007).

**Marker assisted selection:** The explants taken from cotyledonary petioles and hypocotyls of *B. carinata* were used for *Agrobacterium*-mediated transformation. The selectable marker genes were neomycin phosphotransferase II, phosphinothricin acetyl transferase and the reporter gene *β*-glucuronidase, under the control of a tandem 35S promoter (Babic *et al*., 1997). Direct selection on L-phosphinothricin also produced resistant shoots.

## 2.5 ANTHER CULTURE

### 2.5.1 Background and history

Androgenesis is a system of achieving complete plants through *in vitro* culture of anthers or microspore or pollen grains. Male reproductive processes take place in the stamens 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. In androgenesis the normal microspore development or gametophyte pathway is arrested and the pathway is switched to sporophtytic development through direct embryo formation or callus development (Swapan, 2005).

In contrast to apomixis and somatic embryogenesis, which lead to clonal propagation of a specific genotype, androgenic and gynogenic plants reflect the product of meiotic segregation. Thus, they have the characteristic of possessing only one set of chromosomes and therefore are haploid plants. *In vivo* occurrence of androgenic haploids has been reported in *Antirrhinum majus, Crepis tectorum, Hordeum bulbosum, Hordum vulgare, Nicotiana* and *Oenothera scabra* (Sopory and Munchi, 1996).

The first haploid plants *in vitro* were produced from anther culture of *Datura inoxia* (Guha and Maheshwari, 1964) which was a major break-through in the history of haploid plant breeding. After Guha and Masharawi (1964) haploid plants through *in vitro* anther culture in wheat, maize, sugar cane, soybean, rubber, grapes and apple were obtained. In China, several new varieties of rice, wheat, tobacco, and hot and sweet pepper possessing high yield, superior quality, tolerance to abiotic stresses such as cold, early maturity and resistance to diseases have been released through the use of haploids (Kim *et al*., 2004).

Other countries have also used doubled haploid breeding methods for crop improvement. The Republic of Korea released two rice varieties derived from anther culture haploid technique and generally screens annually about 6000 anther-derived rice lines (Chung, 1989). The technique has also been effective in heterosis breeding of Chinese cabbage and in Japan several successful varieties have been developed through this technique (Chen, 1986). Among these rice varieties,

which are tolerant to cold temperature and are good in taste; a cold resistance and uniform shape broccoli variety and a cabbage variety with an orange color were developed by the anther culture method (Moon, 1998).

### 2.5.2 Advantages of androgenesis

The production of haploid and doubled haploid plants via biotechnological approaches such as anther and microspore cultures offers the possibility of accelerating the breeding process as well as facilitating basic scientific research work. In comparison to the conventional breeding methods via repeated self-pollination include reduction of time and space for breeding and ultimately reduction of the costs for cultivars development (Kott, 1998). In addition, the value of doubled haploids in breeding is that they are diploid, fertile and homozygous for all traits, therefore are true breeding in the following generations and each doubled haploid represents a unique combination of traits from each parent in the original cross (Wang *et al.*, 1999). This provides excellent material for research, plant breeding and plant transformation.

Haploid induction through anther or microspore culture was used for production of new varieties, production of homozygous lines for heterosis breeding, selection of stable resistant lines against biotic and abiotic stresses (Bansal, 2002). Recessive alleles in the parent are easily uncovered and genotypes are easier to determine. Haploidy can also be used to produce a male or female plant from dioecious species such as asparagus (Tai, 2003). The populations derived from anther culture have enabled basic researchers to map molecular markers and characterize genetically complex traits while using as a tool for cultivar development (Smykalova *et al*., 2006).

### 2.5.3 Factors influencing androgenesis

Many factors such as genotype, physiological age of donor plant and culture medium affect anther culture reaction for androgenic calli and plant regeneration (Datta, 2005). The findings of Datta (2005) reported that culture medium and genotype were important factors for callus induction from anther culture in rice. Genotype is the most critical factor in obtaining good microsporogenesis irrespective of varieties used under certain culture conditions (Datta *et al*., 1990).

**Donor plants:** which is affected by its age and the environmental conditions under which it has been grown significantly influences the androgenic response (Sunderland and Dunwell, 1974). Generally, buds from the first flush of flowers show better response than those borne subsequently or the anthers excised towards the end of the flowering season exhibit a delayed response and low frequency of sporophyte formation (Thurling and Chay, 1984; Olesen, 1988)

**Temperature shock**: Efficient androgenesis is usually induced by the application of a stress treatment to whole plants *in vivo* or tillers, buds, anthers and isolated microspores *in vitro* (Touraev *et al*., 1997). Temperature shock is found to affect anther or microspore embryogenesis. In many species the incubation of anther cultures at a low temperature (4-5$^0$C) for various periods before shifting them to 25$^0$C enhanced the androgenic response of some plants like barley and Nicotinia (Bhojwani *et al*., 1990). However in others like *Brassica* incubation of the cultured anthers at higher temperature were reported to have a promotory effect. The biological mechanism (s) underlying this developmental switch induced by heat-shock is poorly understood (Zhao *et al*., 2003). Heat shock before or after the culture of intact anthers is reported to have an influence on anther derived embryo formation or plantlet regeneration (Telmer *et al*., 1993), it is believed that the normal developmental pathway of the microspore is switched off and the pathway to develop into an embryo is turned on during androgenesis. For such a process to occur different methods of stresses are used (Zaki and Dickinson, 1990).

**Bud stage:** Cytological studies have shown that bud size could be used as the criterion for cytological readiness of the microspores. Therefore, bud selection is critical for anther culture success (Eyasu Abrha *et al*., 2008).In anther, microspore and pollen cultures the presence of some binucleate grains could be detrimental, pollen population with bicellular grains gave maximum yield of pollen embryos (Telmer *et al*., 1992). The nursing effects of whole anthers for androgenic development introduced the concept of 'wall factor' (Pechan and Keller, 1988).

**Carbohydrate source:** The other factor for androgenenic response is carbohydrate source, all *Brassica* species require as high as 12-13% of sucrose for androgenesis in anther, microspore and pollen cultures. According to Sorvarsi and Schieder (1987) high sucrose concentration favored better survival of pollen grains by improving the frequency of androgenesis in Barley. MacDonald *et al*., (1988) have also reported that most of the cultures of wheat showed higher androgenic responses when sucrose was substituted by maltose in the medium.

**Basal Media:** The difference in media requirements is also considered as a factor affecting androgenic response of plants. Most species exhibit androgenesis on a complete nutrient medium (mineral salts, vitamins and sucrose) with or without growth regulators or growth hormones unlike tobacco and *Datura* (Huang *et al*., 1990). The basal media commonly used are the formulations recommended by Nitsch (1969) or Murashige and Skoog (1962), Gambroge *et al*., (1968), N6 (Chu, 1978) and others.

Others include light which does not seem to be necessary for the induction of embryos or callus in androgenesis, most cultures are kept in dark condition and the effect of gaseous environment on anther culture has been rarely investigated it is considered as one of the factors that affect androgenesis. In addition part of the filament left attached caused a reduction in the androgenic response by about 30% in *Brassica oleracea* (Arnison *et al*., 1990).

## 2.6 Anther culture in *Brassica*

Through anther culture progress has been achieved for a large number of economically important crop species, such as barley, wheat, maize, rapeseed and rice. For the *Brassica* species the first attempt at producing DH (double haploid) lines was made using the anther culture system (Keller and Armstrong, 1977).

After the development of doubled haploids in *Brassica napus* by Keller and Armstrong (1977), anther culture has become a standard technique used in the breeding of oilseed *Brassicas* (Macdonald and Ingram, 1984). The haploids produced often exhibit recessive characters previously masked in the diploid state and may readily be diploidised to produce homozygous lines (Loh and Ingram, 1983).

Microspore culture has been successful in over 250 crop plants including cereals, oilseeds, ornamentals and vegetable crops. Several *Brassica* species have also shown success through microspore cultures (Efftoda, 2002). These include *Brassica oleracea*, *Brassica napus* and *Brassica campestris*. In addition to microspore culture the technique of pollen culture has been considerably improved and androgenic plants through isolated pollen culture have been raised for many crop plants, including *Brassica carinata* (Chuong and Beversdorf, 1985).

The microspore anther doubled haploid (DH) methodology is now employed in many *Brassica* breeding programmes around the world as an alternative to conventional methods of homozygous line production (Kott and Beversdorf, 1990; Chen *et al*., 1994; Ferrie and Keller, 1995; Burbulis *et al*., 2004).

Application of heat stress pre-treatment or heat shock immediately after anther plating promoted embryo induction in *Brassica* species (Custers *et al*., 1996). Heat shock treatments with different temperature regimes were followed since heat stress pre- treatment is an essential factor to increase the efficiency of androgenesis in most *Brassica* species.

Considerable effort has recently been exerted to increase anther culture efficiency in *Brassica napus,* largely by the use of heat-shock treatments during the first few days of culture (Keller and Armstrong, 1977; Dunwell, *et al.,* 1983; Angadi *et al*., 2000). Such treatments have been very effective in increasing the embryo production potentials of some *Brassica* species (Barro and Martin, 1999).

The development of microspore culture techniques in *Brassica* has resulted in increased embryo yields through efficient procedure (Chuong and Beversdorf, 1985). But anther culture is a valuable starting point for studies of haploids because of its relatively simple method of isolation and minimal facility requirements (Jahne and Lorz, 1995). Anther culture also is important experimental tool to investigate plant regeneration, ploidy level of anther derived plantlets and the factors affecting the process of androgenesis (Macdonald *et al*., 1988). This study is done in an attempt to investigate some of the major factors influencing anther culture of *Brassica carinata*.

# 3. OBJECTIVES

## 3.1 General objective

The general objective of the study is to regenerate plants from anther culture of *Brassica carinata* (Ethiopian mustard).

## 3.2 Specific objectives

The specific objectives of the study are the following

- To identify the appropriate bud stage for anther isolation and culture.
- To optimize heat treatment for embryo induction.
- To investigate the appropriate medium for embryo induction.
- To investigate the effect of growth regulators on plantlet regeneration.
- To identify optimum growth regulator concentration for root development.
- To evaluate survival rates of acclimatized plant regenerants.

# 4. MATERIALS AND METHODS

## 4.1 Plant Materials

Four varieties of *Brassica carinata* namely Holeta 1, PGRCE07/1/2, S-67 and Yellow Dodola were obtained from Holeta Agricultural Research Institute, Highland Oil Crops Research Department. Seeds of 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. Seeds of the four varieties are shown in Fig 2.

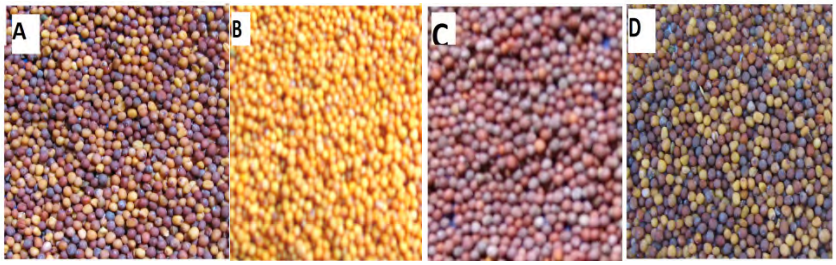


Fig 2. Seeds of *Brassica carinata* varieties. (A) Holeta 1,(B) Yellow Dodola, (C) S- 67 and (D) PGRCE 07/1/2

## 4.2 Stock solution and culture media preparation

The stock solution of the four media, namely, NN (Nitsch and Nitsch, 1969), as modified by Keller *et al*., (1995), MS (Murashige and Skoog, 1962), $B_5$ (Gamborg *et al*., 1968) and $N_6$ (Chu, 1978) were prepared by dissolving the appropriate amount of macro and micro nutrient; and organic supplements (Appendix I Table 1-4). Similarly, growth regulators stock solutions for BAP, IAA, IBA, $GA_3$ and NAA were worked out using the proportion of 1mg: 1ml. The stock solutions of basal media and plant growth regulators were stored in refrigerator at $4^0$C and deep-freezer at -$20^0$C, respectively for a month.

The four culture media were prepared from their respective stock solutions using the appropriate amount of sucrose, plant growth regulators and agar (7 g/l). The media were steam sterilized using autoclave at a temperature of $121^0$C with a pressure of 0.15 Kpa for 15 minutes.

## 4.3 Grouping of flower buds into appropriate stages for anther culture

Bud stage analysis was carried out to determine the appropriate bud stage where the maximum number of responsive anthers can be isolated. Twenty flower bud samples from each variety were taken and their lengths were measured using a caliper. The buds were categorized into three length ranges and the most dominant anther colors associated with each bud length were determined (Table 2 and Fig 3).

Table 2. Grouping of buds into three stages according to bud length and anther color

| **Bud stage** | **Bud length** | **Anther color** |
|---|---|---|
| **$B_1$** | 1.5 – 2.5 cm | Deep Green |
| **$B_2$** | 2.5 – 3.5 cm | Light green |
| **$B_3$** | 3.5 – 4.5 cm | Greenish yellow |


Fig. 3 The three bud stage categories. (B1) Bud stage 1, (B2) Bud stage 2 and (B3) Bud stage 3

## 4.4 Bud collection and surface sterilization

Immature flower buds were harvested from the first flushes of flower buds grown in the glasshouse. Surface sterilization of the collected buds was achieved in 70% alcohol for 45 seconds followed by 5 % potassium chloride for 10 minutes. The buds were rinsed 4 times with double distilled sterilized water before excision of intact anthers.

## 4.5 Anther excision and plating

The sterilized flower buds were placed under stereomicroscope and anthers along with their filaments were excised under aseptic condition. Six anthers from each bud taken from all varieties were gently detached from their filaments without injuring the anthers. Anthers were plated horizontally on10cm Petri-dish containing 30ml solidified culture media supplemented with the required concentrations of plant growth regulators.

## 4.6 Identification of appropriate bud stage

Ten anthers from each varity were cultured on $B_5$ medium supplemented with 0.5 mg/l BAP and 1 mg/l NAA for experiment to identify the appropriate bud stage for maximum embryo induction. A Petri-dish with 10 anthers was a unit of replication and there were three replications.

## 4.7 Determination of the effect of temperature duration on embryo induction

Five flower buds from each of the three bud stages were taken for all the four varieties and plated on the four culture medium (NN, MS, $B_5$ and $N_6$), which contained 1mg/l NAA and 0.5mg/l BAP with 13% (w/v) sucrose and 7g/l agar each. The cultured anthers with three replications (10 anthers/ Petri-dish) were treated under four temperature regimes to investigate the effect of temperature treatment on embryo induction. The heat shock treatments were the following; H0 – control, the cultured anthers were kept in dark continuous culture condition at a temperature of $25^0$C, H1- those cultured anthers incubated at 32 $^0$C for 24h period then transferred to the dark continuous culture condition at $25^0$C, H2 – those cultured anthers that incubated at 32 $^0$C for 48h

period then transferred to the dark continuous culture condition at $25^0$C and H3 - those cultured anthers that were incubated at $32^0$C for 72h period then transferred to the dark continuous culture condition at $25^0$C.

## 4.8 Effect of basal culture media difference on embryo induction

Anthers excised from all the three bud stages of the four varities were cultured on 30ml of the sterilized NN, $B_5$, MS and $N_6$ media supplemented with 1 mg/l NAA and 0.5 mg/l BAP with 13% (w/v) sucrose and 7g/l agar. Ten anthers were cultured per Petri-dish and three Petri-dishes for each treatment and a total of 144 Petri-dishes and 1440 anthers were investigated for embryo induction.

## 4.9 Embryo germination and plantlet regeneration

After embryos started to develop, the cultures were transferred for germination on $B_5$ medium supplemented with 0.2mg/l BAP and 0.2mg/l IAA, 2% (w/v) sucrose and 7g/l agar and kept in the culture room at a temperature of $25^0$C $\pm$ $2^0$C under 16h of light (2700 lux light intensity) and 8hrs of dark.

Then the embryos were transferred to $B_5$ regeneration medium with four growth regulator combinations to investigate the effect of growth regulators on plantlet regeneration. The growth regulator combinations used were, Treatment 0, $B_5$ medium without growth regulators or control; treatment 1, $B_5$ medium supplemented with 0.02mg/l BAP and 0,01mg/l NAA; treatment 2, $B_5$ medium supplemented with 0.01mg/l BAP and 0.02mg/l NAA and treatment 3, $B_5$ medium supplemented with 0.01mg/L $GA_3$.

Five embryos were transferred to a magenta box containing 40ml of $B_5$ medium with different plant growth regulator combinations and the control as well. A magenta jar was a unit of replication with 5 replications for each treatment. The plantlets were sub-cultured every two weeks till root development and were kept at the same condition.

## 4.10 Root development and acclimatization

Root development experiment was conducted on $B_5$ medium supplemented with two concentration of IBA (0.01 and 0.02 mg/l) including the control ($B_5$ medium without IBA) to evaluate the effect of plant growth regulators on root development.

Plantlets with approximate height of 7-8 cm, with well developed roots of 4-5 cm length, were taken from the culture jar using forceps and transferred to small acclimatization pots measured a height of 7cm and a width of 4cm containing a sterilized soil mix with a proportion 50% red soil: 25% sand and 25% compost respectively. Each pot was covered with plastic bag and kept in the growth room at $25^0$C $\pm$ $2^0$C 16hrs of light (2700 lux light intensity). After a week of acclimatization, plantlets were transferred to green house with 80% humidity, $25^0$C temperature and 16h light of about 2700 lux of light intensity and 8h dark by removing the plastic bag and regenerants were grown to maturity.

## 4.11 Experimental design and data analysis

The experimental design used for this study was completely randomized design (CRD). Statistical analysis of quantitative data was carried out by JMP SAS version 8.0 software and excel spread sheet. Difference at probability level of $p \leq 0.05$ was considered significant for all analysis.

# 5. RESULTS

## 5.1 Bud stage dependant embryonic responses

Morphological staging system was identified by measuring the flower bud length using a caliper to determine the stage at which maximum number of embryos induced from cultured anthers (Table. 2). According to the preliminary anther culture experiment, the best embryo response was achieved from the anthers isolated from flower buds of bud stage 1, with a length range of 1.5-2.5mm followed by bud stage 2 flower buds measuring 2.5-3.5 mm in length (Fig. 4) for all varieties and basal media used. While bud stage 3 or those flower buds with the length range of 3.5-4mm were the least responsive from the three bud stages used. Based on the analysis of variance it was found that the different genotypes and bud stages significantly affected embryo induction. The effect of the two factors, however, is independent since their interaction is statistically non-significant ($P \leq 0.05$).

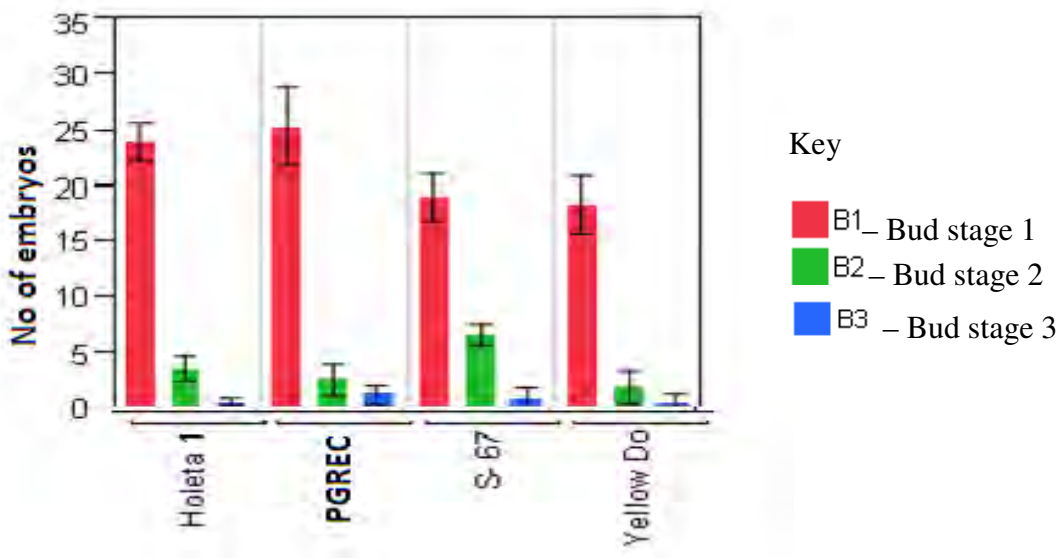


Fig 4. Embryogenic response of the varieties Holeta 1, Yellow dodola, S – 67 and PGREC 07/1/2 at different bud stages (B1= bud stage 1, B2=bud stage 2 and B3= bud stage 3). Each error bar is constructed using 1 standard error from the mean.

Table 3. ANOVA for genotype response to the three bud stages

| Source | DF | Sum of squares | F ratio | Prob>F |
|---|---|---|---|---|
| Genotype | 3 | 111.3333 | 4.2145 | 0.0158* |
| Bud stage | 2 | 3038.3889 | 172.5268 | <.0001* |
| Genotype X Bud stage | 6 | 108.9444 | 2.0620 | 0.0961 |
| Error | 24 | 211.3333 | | |

## 5.2 The effect of duration of incubation temperature on embryo induction

The type of stress used for embryo induction was heat shock and the results indicated that, highest number of embryo was obtained with 24h incubation period at $32^0$C immediately after inoculation for all the four varieties (Fig. 5). Other temperature regimes showed less embryo induction compared to heat treatment 1 (H1). The results from the effect of different temperature regimes on embryo induction were presented in the appendix II (Table 2). The analysis of variance for the effect of temperature incubation duration on embryo induction is presented in (Table 4). According to the analysis of variance genotype and duration of incubation temperature immediately after anther culture significantly affected the embryo induction. The effect of differences in genotype was found to be statistically non-significant for the effect of temperature treatment on embryo induction.

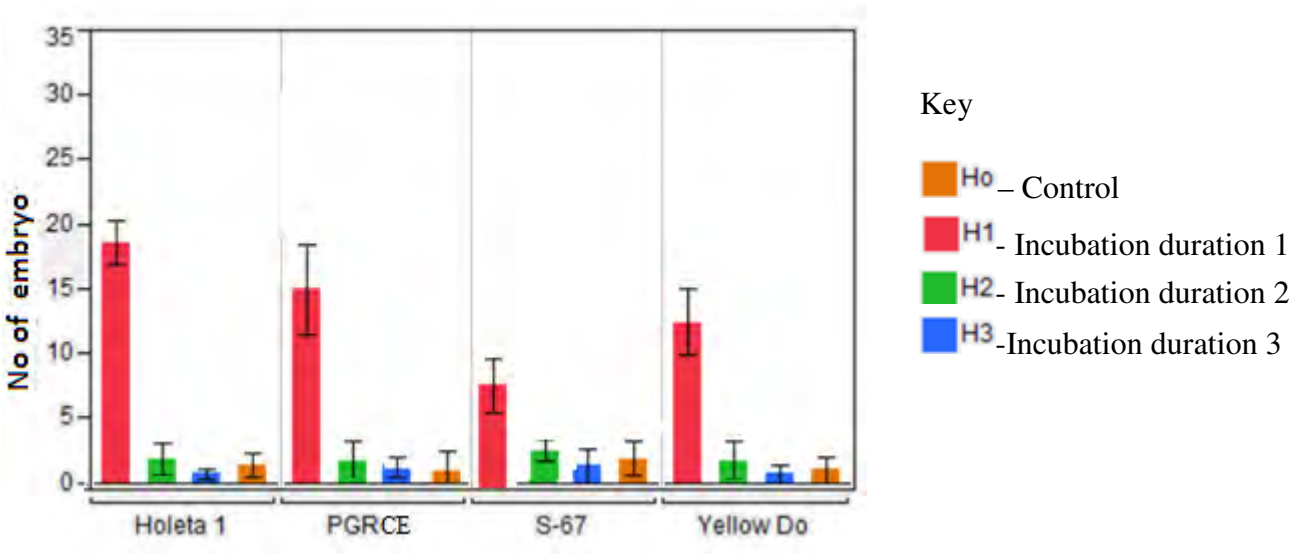


Fig.5 Embryo induction of varieties Holeta 1, Yellow Dodola, S – 67 and PGREC07/1/2 at $32^0$C for different incubation duration (H0= control, H1= incubation duration 1, H2= incubation duration 2 and H3 incubation duration 3). Each error bar is constructed using 1 standard error from the mean.

Table 4. ANOVA for the effect of heat treatments on embryo induction response by different genotype s

| **Source** | **DF** | **Sum of squares** | **F ratio** | **Prob>F** |
|---|---|---|---|---|
| Genotype | 3 | 111.3333 | 4.7502 | <.00075* |
| Heat treatment | 3 | 3460.2292 | 147.6364 | <.0001* |
| Genotype X Heat treatment | 9 | 115.5208 | 1.6430 | 0.1450 |
| Error | 32 | 250.0000 | | |

## 5.3 The effect of different basal media on embryo induction

Embryo induction started 3-4 weeks after culture. Results of the embryo induction experiment are presented in the appendix II (Table 2) in text Table. According to these results, the maximum mean numbers of embryos were obtained on $B_5$ medium supplemented with 0.5 mg/l BAP and 1 mg/l of NAA, 13% sucrose (w/v) and 7g/l of agar for all varieties except Holetta 1. Statistically significant ($p \leq 0.05$) highest mean number of embryos was 41.3, 36.3 and 26.0 for PGRCE07/1/2, Yellow Dodola and S-67, respectively (Table 5). Unlike the three varieties, Holetta 1 scored the maximum mean number of embryos (40.3) on NN medium supplemented with 0.5 mg/l BAP and 1 mg/l of NAA. $N_6$ basal medium supplemented with the same growth regulators concentration showed the least embryo response for all the varieties compared to the other basal media used. Over all variety Holeta 1 was found to the most responsive giving maximum embryo numbers on two basal media namely NN and $B_5$.

According to analysis of variance (Table 6), both genotype and basal media showed highly significant ($p \leq 0.05$) effect on embryo induction. The effect of the interaction of the two factors was also highly significant at $p \leq 0.05$.

Table 5. Mean comparison for the effect of basal media difference on embryo induction

| Media | Mean number of embryos | | | |
|---|---|---|---|---|
| | **Holeta 1** | **Yellow Dodola** | **S-67** | **PGRCE07/1/2** |
| NN | $40.3 \pm 1.5^{a}$ | $15.0 \pm 2.0^{b}$ | $8.3 \pm 1.5^{bc}$ | $2.3 \pm 1.5^{c}$ |
| $B_5$ | $36.3 \pm 2.1^{a}$ | $40.6 \pm 3.5^{a}$ | $26 \pm 2.0^{a}$ | $41.3 \pm 3.1^{a}$ |
| MS | $19.0 \pm 2.0^{b}$ | $14.6 \pm 1.5^{b}$ | $10.3 \pm 3.5^{b}$ | $21.3 \pm 3.5^{b}$ |
| $N_6$ | $4.3 \pm 1.5^{c}$ | $1.0 \pm 1.0^{c}$ | $4.3 \pm 1.5^{c}$ | $2.0 \pm 1.0^{c}$ |

Levels not connected by the same superscript letters in the same column are not significant at $p \leq 0.05$.

Table 6. ANOVA for the effect of basal media difference on embryo induction

| Source | DF | Sum squares | F ratio | Prob>F |
|---|---|---|---|---|
| Genotype | 3 | 2513.0000 | 177.1278 | <.0001* |
| Media | 3 | 6714.4167 | 473.2628 | <.0001* |
| Genotype X Media | 9 | 2198.4167 | 51.6515 | <.0001* |
| Error | 32 | 151.333 | | |

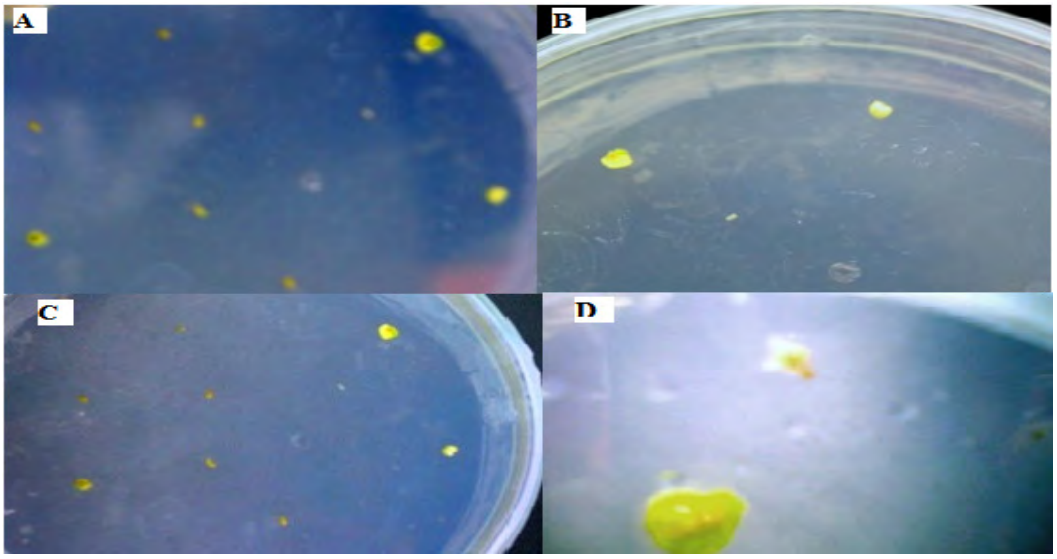


Fig.7. Twenty eight days old embryos (A) Holeta 1, (B) PGRCE07/1/2, (C) S – 67 and (D) Yellow Dodola

## 5.4 Embryo germination and Plantlet Regeneration

All embryos were transferred to $B_5$ germination medium supplemented with 0.2mg/l BAP and 0.2mg/l IAA, germinated embryos showed a noticeable size increase after 21 days (Fig.8).

All the 255 germinated embryos were transferred to $B_5$ regeneration medium with four growth regulator combinations named as Treatment 0 $B_5$ medium without growth regulators or control, Treatment 1 $B_5$ medium supplemented with 0.02mg/l BAP and 0.01mg/l NAA; Treatment 2 $B_5$ medium supplemented with 0.01mg/l BAP and 0.02mg/l NAA and Treatment 3, $B_5$ medium supplemented with 0.01mg/l $GA_3$ with 5 replications each. And the results revealed that the embryos on Treatment 0 showed plantlet growth after 2 weeks (Fig. 9 and Fig. 11), where as the three growth regulator supplemented media failed to give plantlets and most of the embryos developed into callus (Fig. 10). Subsequent culturing every two weeks were carried out and plantlets showed better development (Fig. 12) till they develop roots. The analysis of variance showed that growth regulator treatment difference significantly affected plantlet regeneration. While the genotype difference and the effect of plant growth regulators on plantlet regeneration was found to statistically not significant (Table 7).

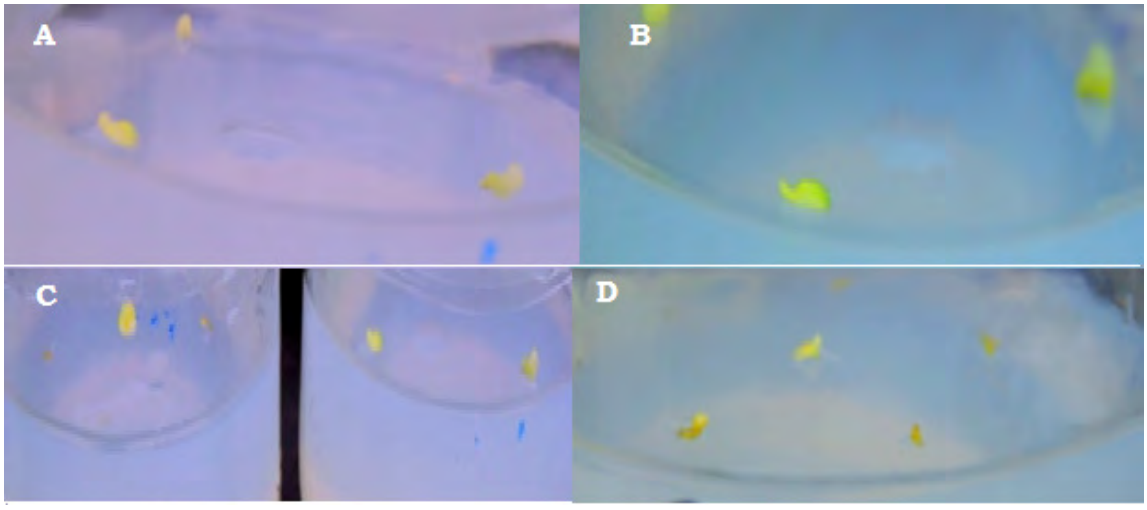


Fig.8 Germinated embryos on $B_5$ medium supplemented with 0.02mg/L BAP and 0.2mg/L NAA, after 21 days of transfer to germination medium. (A) Holeta 1, (B) PGRCE07/1/2, (C) S – 67 and (D) Yellow Dodola

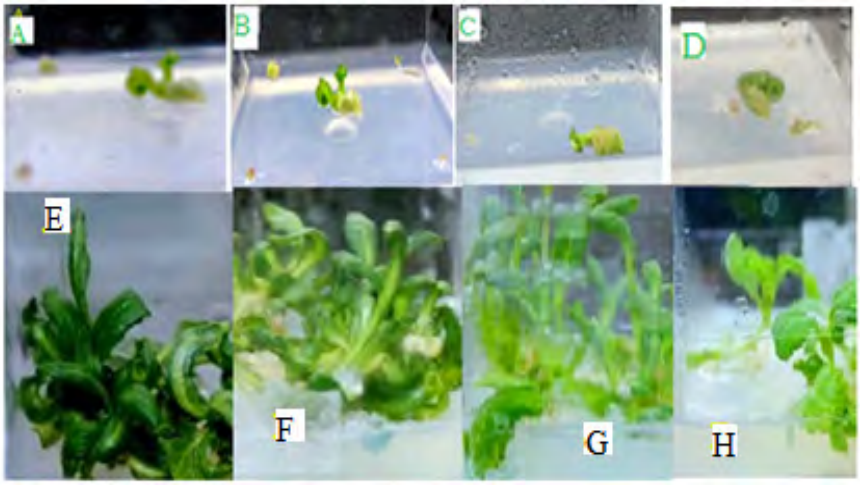


Fig 9. Plantlet regeneration (upper row) and plantlet growth (lower row)of the four varieties on $B_5$ medium not supplemented with growth regulators (A & E) Holeta 1, (B & F) PGRCE07/1/2, (C & G) S- 67 and (D & H) Yellow Dodola.

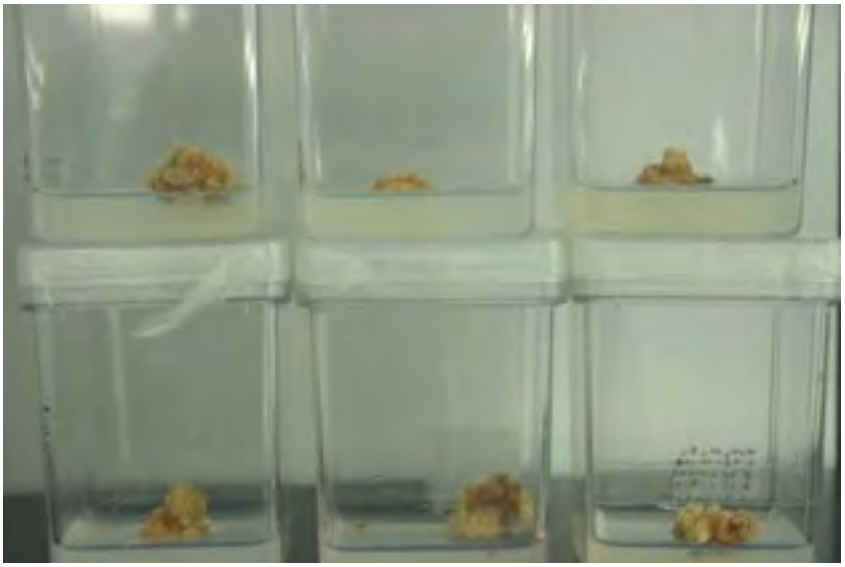

Fig 10. Calli on $B_5$ regeneration medium supplemented with growth regulators

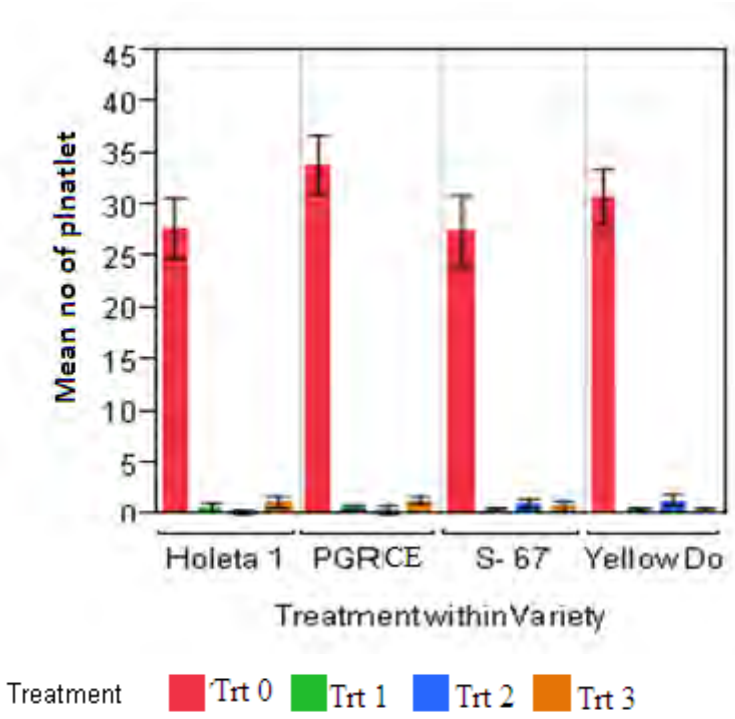


Fig.11 The effect of different growth regulator concentrations for plantlet regeneration on $B_5$ basal medium(Trt 0= $B_5$ without growth regu;ator, Trt 1= $B_5$ with 0.02mg/l BAP and 0.01mg/l NAA, Trt 2= $B_5$ with 0.01mg/l BAP and 0.02mg/l NAA and Trt 3= $B_5$ with 0.01mg/l $GA_3$) . Each error bar is constructed using 1 standard error from the mean.

Table 7. ANOVA for plantlet regeneration on $B_5$ medium

| **Source** | **DF** | **Sum squares** | **F ratio** | **Prob>F** |
|---|---|---|---|---|
| Genotype | 3 | 136.150 | 3.8113 | 0.0143* |
| Media | 3 | 12748.367 | 356.8714 | <.0001* |
| Genotype X Media | 9 | 106.070 | 0.9898 | 0.4577 |
| Error | 62 | 738.267 | | |


Fig. 12. Forty five to fifty days old plantlets sub cultured on to growth regulator free $B_5$ medium (A & E) Holeta 1, (B & F) PGRCE07/1/2, (C & G) S – 67 and (D & H) Yellow Dodola. Each genotype is represented by two plantlets.

## 5.5 Root development

After a month and half on the regeneration media all plantlets were transferred to $B_5$ medium supplemented with 0.01 and 0.02mg/l IBA and without growth regulators as control to identify the effect of IBA on further root development of the plantlets. According to the results obtained from root development experiment, the plantlets transferred to IBA supplemented media developed callus (Fig, 13) while the plantlets transferred to growth regulator free $B_5$ medium showed root development. Noticeable root length increases of 1.5-2cm were observed after 15 days (Fig.14). The root counts showed that average numbers of roots were found to be 8.5 for Holeta 1, 8.25 for PGRCE07/1/2, 10.25 for S – 67 and 9.75 for Yellow Dodola with no significant difference among the varieties.

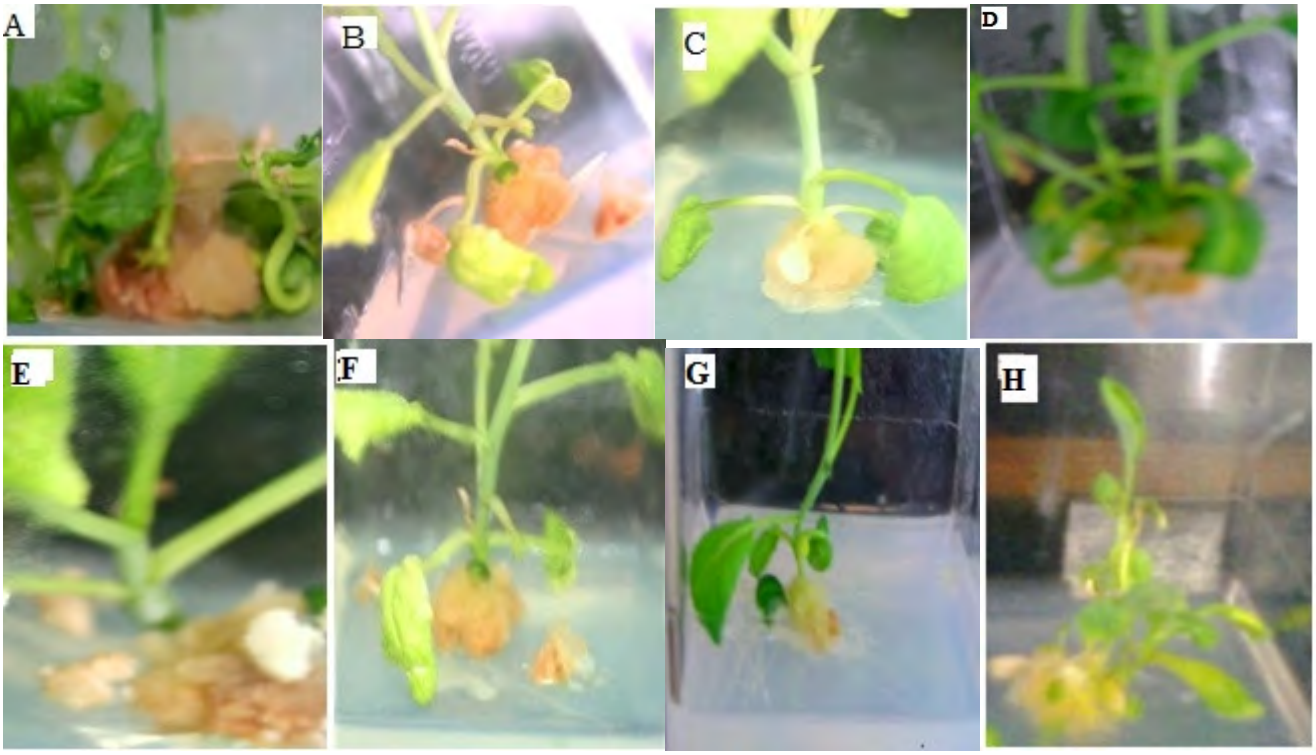


Fig.13. Plantlets on rooting media supplemented with 0.01mg/L IBA after two weeks of transfer (A, B, C, D) and 0.02mg/L IBA (E, F, G, H). (A & E) Holeta 1, (B & F) PGRCE07/1/2, (C & G) S – 67 and (D & H) Yellow Dodola.


Fig.14 Root development on $B_5$ medium without growth regulators (A) Holeta 1, (B) PGRCE07/1/2, (C) S – 67 and (D) Yellow Dododla. Four plantlets are shown for each variety.

## 5.6 Acclimatization

A total of 121 plantlets; 38 Holeta 1, 31 PGRCE07/1/2, 25 S – 67 and 27 yellow Dodola were acclimatized on a sterile soil mix containing 50% red soil: 25% sand: 25% compost on acclimatization pots (7cm length and 4cm width) covered with plastic bags (Fig. 15). After a week in the growth room the plastic bags were removed (Fig. 16) and the plantlets were transferred to a green house with 80% humidity, at a temperature of 25$\pm$ $2^0$C and 16hrs light and 8hrs of dark. After a month in the green house the survival rates were recorded (Table 8) and the survival rates were found to be, 100% for varieties PGRCE07/1/2 and S – 67; and 97.37% and 96.3 for Holeta 1 and Yellow Dodola respectively. There were no aberrant phenotypes for all the varieties grown in the green house (Fig. 17). The plantlets flowered after 2 months in the green house (Fig.18).

Fig. 15. Sixty five to seventy day old, 7-8cm high plantlets acclimatized in the growth room covered with plastic coverings.


Fig.16. Acclimatized plantlets in the growth room after a week of acclimatization (A)Holeta 1, (B) PGREC 07/1/2, (C)S – 67 and (D)Yellow Dodola

**Table 8. Survival rate of acclimatized plantlets in the growth room and green house**

| Variety | Number of plantlets Transferred to pots | Transferred to greenhouse | Survived in the greenhouse (%) |
|---|---|---|---|
| Holeta 1 | 38 | 38 | 37(97.3%) |
| PGRCE07/1/2 | 31 | 31 | 31(100%) |
| S-67 | 25 | 25 | 25(100%) |
| Yellow Dodola | 27 | 27 | 26(96.3%) |


Fig.17. Forty five days old hardened plantlets in the green house (Left) (A) Holeta 1, (B) PGRCE07/1/2 (C) S – 67 and (D) Yellow Dodola. (Right) 6 weeks' old plantlets in the greenhouse.

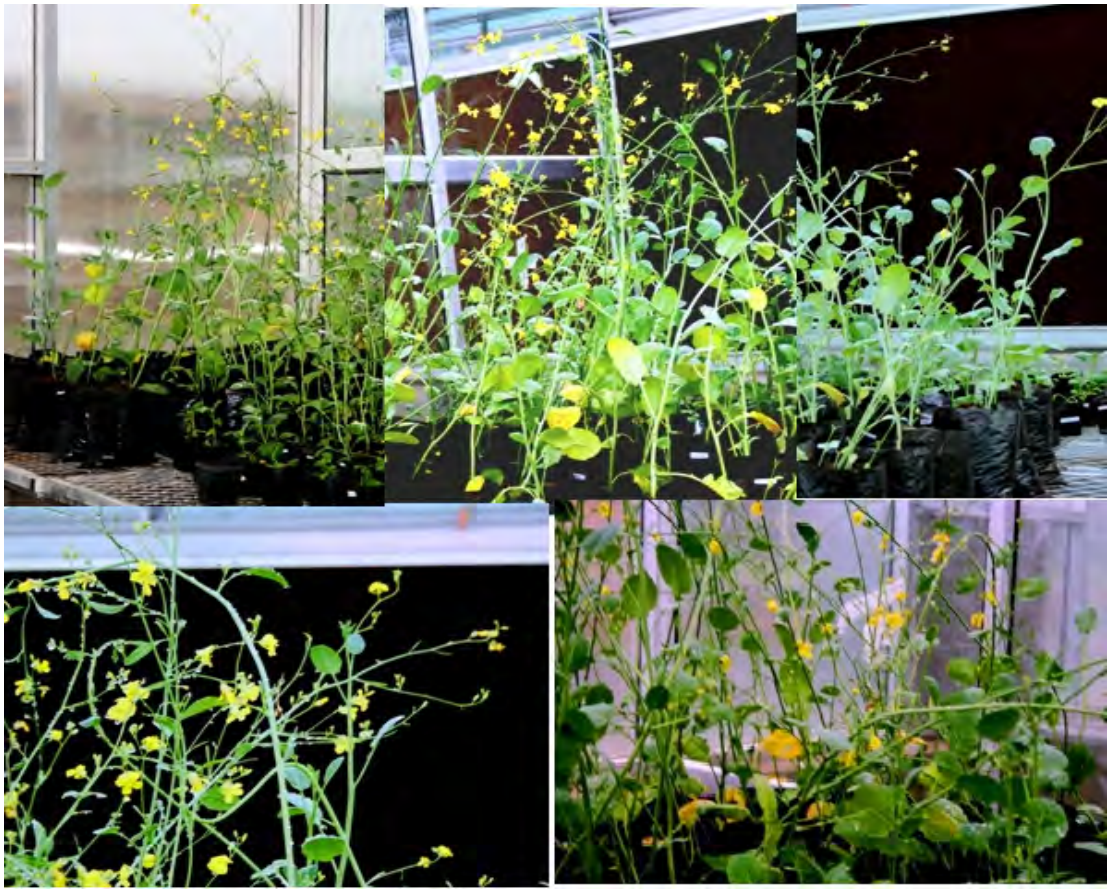

Fig 18.  Anther derived plantlets flowering after a month and half in the green house.

# 6. DISCUSSION

## 6.1 Bud stage determination

Bud length is an easy parameter in order to select flower buds at the right nucleate stage for increasing embryo induction in androgenesis (Olesen, 1988). In this study morphological staging system was used and three bud stages were identified by measuring the bud length using a caliper. The identified bud stages were with the following length ranges; bud stage 1(1.5 – 2.5 mm), bud stage 2(2.5 – 3.5mm) and bud stage 3(3.5 – 4.5mm). The anthers isolated from bud stage 1 were deep green in color, very young and too small to isolate; the anthers isolated from bud stage 2 were light green in color and easy to isolate while the anthers from bud stage 3 are larger with no difficulty to isolate and gave the least number of embryos compared to the two bud stages used. The results from the preliminary experiment done showed that bud stage 1 or flower buds with a length ranging from 1.5- 2.5mm were found to be more responsive for all varieties. The finding of the study is in agreement with Eyasu Abrha *et al* (2007) for microspore culture of *Brassica carinata* and Smykalova *et al* (2003) in the microspore development of *Brassica carinata*. The bud stages with length exceeding 3.5mm and above were unresponsive, this could be attributed to the production of toxic substances by larger microspore cells that inhibit cell division and embryogenesis (Gil-Humanes and Barro, 2009). According to Gil-Humanes and Baro (2009) the most recommended bud sizes are those at the early stage development with the size range of 1.5-3.0mm in length.

## 6.2 The effect of temperature incubation duration on embryo induction

Application of various stress mechanisms strongly influenced switching the gametophytic pathway of microspore development to sporophytic pathway of development to get haploid and doubled haploid plants (Touraev *et al*., 1997). According to Zhao *et al* (2003) Post culture incubation at higher temperature has been reported to improve androgenetic response in *Brassica* species. In the present study the maximum number of embryos was achieved when the anthers were incubated at the temperature of $32^0$C for 24hrs immediately after inoculation in the dark

culture condition then transferring them to $25^0$C continuous culture condition till the induction of embryos, this result is in consistence with Barro and Martin (1999). After the embryos were induced the culture were transferred into light cycle. Choung and Berversdof (1985) also reported that, incubation of cultured microspores at a temperature of $32^0$C for 1-3 days immediately after culture increased embryogenesis in the microspore culture of *Brassica carinata* and *Brassica napus.* The temperature regime or the incubation period identified ($32^0$C for 24h) was the best for all the varieties compared to the other temperature regimes investigated for the study.

## 6.3 The effect of basal media on embryo induction

Different researchers have used different media for regeneration of plants from androgeneic embryos. Keller *et al* (1975) used $B_5$ and MS media without hormones with 2% (w/v) sucrose for *Brassica campestris* and *Brassica napus.* Keller (1991) used $B_5$ medium without hormones for *Brassica oleracea* embryo culture and obtained either direct development of embryos into plants or shoot induction after several passes. Similar results with these media were also obtained by Lelu and Bollon (1990) for *Brassica oleracea* var. *capitata* and *Brassica oleracea* var. *gemmifera*.

Medium composition determines the success of plantlet regeneration from androgeneic embryos, since nutrient media not only provides nutrition to the anthers but also directs the pathway of embryo development (Keller *et al.,*1975). Four types of media with the same growth hormone concentration (1mg/l NAA and 0.5mg/l BAP) and same sucrose concentration 13% (w/v) were used for embryo induction. The maximum embryo numbers were achieved on $B_5$ media supplemented with 1mg/l NAA and 0.5mg/l which is in harmony with the findings of Roy and Saha (2006).

Nutrient media is highly genotype dependent and also in this study it was found that there was genotype difference in embryo response of the three varieties, and PGRCE07/1/2, S-67 and Yellow Dodola manifested maximum embryo number of 41.3, 26.0 and 36.3, respectively on $B_5$ medium. This result agrees with the result reported by Efftoda (2002). Unlike the preceding three

varieties, Holeta 1 showed its maximum embryo number (40.3) on NN medium and this result is in agreement with that of Eyasu Abrha *et al* (2008). According to their finding, NN medium was suitable for microspore embryogenesis of *Brassica carinata*.

The other factor that greatly influences embryogenic responsivity of androgenesis is carbohydrate source or sucrose concentration, which, besides being a carbon source, regulate medium osmolality (Gil-Humanes and Barro, 2009). In the present study 13% sucrose (w/v) was used for all the media as recommended by Efftoda (2002); Eyasu Abrha *et al* (2008); Barro *et al* (2001). The best working pH for the media preparation was found to be 5.8 as suggested by several authors like Nitich (1988); Lichter (1982); Efftoda (2002); Eyasu Abrha *et al* (2008) and Barro *et al* (2001).

## 6.4 Embryo germination and plantlet regeneration

The induced embryos were transferred to embryo germination $B_5$ medium supplemented with 0.2mg/L BAP and IAA 2% (w/v) sucrose and 5.8 pH for 16h of light and 8hrs of dark (200lux) at a temperature 25 $\pm$ $2^0$C. As suggested by Efftoda (2002) and Eyasu Abrha *et al* (2007), all the embryos germinated or increased in size from 0.5mm to 1.5 mm in diameter after 3 weeks on embryo germination medium.

For regeneration of plantlets the best recommended media are those media without addition of growth regulators. Keller *et al* (1975) used $B_5$ and MS media without hormones with 2% (w/v) sucrose for *Brassica campestris* and *Brassica napus.* Keller (1991) used $B_5$ medium without hormones for *Brassica oleracea* embryo culture and obtained either direct development of embryos into plants or shoot induction after several passes. In the present study, most of the germinated embryos transferred to a regeneration media produced plantlets on $B_5$ medium devoid of growth regulators containing 2%(w/v) sucrose at a pH of 5.8. This is harmonious with Eyasu Abrha *et al* (2008) for microspore culture of *Brassica carinata*. For plantlet regeneration growth hormone free medium was most responsive according to the findings of Macdonald *et al* (1988) in the anther culture of *Brassica napus* which was also coherent with the results of this study.

The hormone combinations applied to investigate the effect of growth regulators on plantlet regeneration in the study were according to the suggestions by Ferrie *et al* (2008); Gil- Humanes and Barro (2009) $B_5$ medium supplemented with different BAP and NAA combinations (Treatment 1 and Treatment 2); and Barro *et al* (2001) reported that 0.01mg/l GA3 (Treatment 3 in the study) showed better plantlet regeneration in *Brassica hirta* microspore culture. However, the three growth regulators combination suggested by the mentioned authors for different *Brassica* species gave the least plantlet development, almost all of the embryos transferred to the above medium with different growth regulator combination developed in to callus.

The genotype difference was found to be statistically non significant in affecting plant regeneration as the growth regulator difference on $B_5$ medium, which significantly affected plantlet regeneration. The results were coherent with the findings of Gorecka and Krzyzanowska (1990). Sub culturing of the plantlets for full development was done on $B_5$ media every 2 weeks before the transfer of the plantlets onto a rooting medium as recommended by Eyasu Abrha *et al* (2008).

## 6.5 Root development and acclimatization

The effect of IBA on root development at two concentrations (0.01mg/l and 0.02mg/l) and growth regulator free $B_5$ medium as a control were considered for the study. The observations from root development potential of the four varieties were considered. It was found that root development of the plantlets was best achieved on $B_5$ media without growth regulators than IBA supplemented $B_5$ medium. The plantlets transferred to growth regulator free $B_5$ medium showed a root length increase of 1.5-3cm after two weeks. The work by Efftoda (2002) reported growth regulator free $B_5$ media promoted rooting of *Brassica napus* which agrees with the present finding. The result was also in consistence with the finding of Gil-Humanes and Barro (2003).

Anther derived or other *in vitro* developed plantlets are fragile till the physiological adjustments fit to the surrounding environment. Plantlets of the four varieties, Holeta 1, PGRCE, S – 67 and Yellow Dodola were acclimatized in the growth room on a sterile soil mix covered with plastic bags to prevent desiccation. After a week the plastic coverings were removed and the plantlets

were transferred to the green house and the survival rates were observed after 30 days of development in the green house. The highest plantlets survival rate was found to be 100% for variety PGRCE07/1/2 and S – 67 and 97.3 and 96.3% for Holeta 1 and Yellow Dodola respectively. The plantlets showed successful survival rates in the greenhouse due to its favorable conditions. Only two acclimatized plantlets died in the greenhouse, one from Holeta 1 and the other from variety Yellow Dodola.

# 7. CONCLUSION

The appropriate bud stage for anther isolation was identified, the flower buds with a length range of 1.5- 2.5mm were more responsive than the other 2 bud stages considered for the study. Statistically no significant difference was observed for the response of all the varieties used.

Heat shock treatment was recommended to promote embryo induction in most *Brassica* species, In the present study, the incubation of cultured anthers at $32^0$C for 24h was found to be the best temperature incubation duration for all the varieties included in the study.

From the four basal media considered for the experiment, the maximum numbers of embryos were achieved on $B_5$ medium supplemented with 1mg/l NAA and 0.5mg/l BAP followed by MS and NN media. Statistically, the basal medium and genotype affected embryo induction independently.

According to the findings of the present study, germination of embryos were successful on $B_5$ medium supplemented with 0.2mg/l BAP and 0.2mg/l IAA. For plantlet regeneration and root development, growth regulator-free $B_5$ medium was found to be the best.

Very high survival rates for all the varieties were achieved by using sterile soil mix in the green house. Varieties PGRCE07/1/2 and S – 67 showed 100% survival and Holeta 1 where as Yellow Dodola showed 97.3% and 96.3% survival rate, respectively.

# 8. RECOMMENDATIONS

There are about 1549 anther derived regnerants produced in the present study. They would need a follow up and screening to make sure the utilization of the anther derived regenerants in selection and future improvement of the crop.

Investigating factors affecting the process of androgenesis at the genetic level and studying heritability of characters through the application of molecular techniques is important.

The factors studied by this experiment are only the effects of bud size, basal medium and duration of incubation temperature on embryo response. It is recommended that further study on the other factors controlling anther culture success in *Brassica carinata* is necessary.

Other protocols like multiplication and node culture of the regenerated plants *in vitro* should be considered to increase the number of plantlets regenerated and to facilitate selection efficiency.

The anther derived plantlets could be haploid, diploid, doubled haploid or polyploidy. The parameters influencing the ploidy difference should be studied. Determination of the ploidy level for the anther derived plantlets is important in order to isolate and use haplpoid regenerants in the breeding of *Brassica carinata*.

## 9. REFERENCES


Adefris Teklewold and Nigussie Alemayehu (1996). Agro-ecology, distribution and improved production technologies of the highland oil crops in Ethiopia. **In:** Abera D. and Beyene S. (eds). Research achievements and technology transfer attempts: Vignettes from Shoa. Proc. of the First Technology Generation, Transfer and Gap Analysis Workshop.25-27 Dec., 1995, Nazareth, Ethiopia, pp. 38-48.

Al-khatib, K. and Boydston, R. A. (1999). Weed control with *Brassica* green manure crops. ***In:*** S.S. Narwal (Ed.), *Allelopathy Update, Basic and Applied Aspects*. New Delhi: Oxford and IBH Publishing Co. Chatham, UK: Volume 2. pp. 255-270.

Angadi, S.V., Cutgorth H.W., Miller R.R., McConkey B.G., Entz M.M., Brandt S.A. and Volkmar K. M. (2000). Response of three *Brassica* species to high temperature stress during reproductive growth. *Can. J. Plant Sci.* 80: 693–701.

Arnison P.G., Donaldson P., Ho L.C. and Keller W.A (1990). The influence of various physical parameters on anther culture of Broccoli (*Brassica oleracea* var. *italica*). *Plant Cell, Tissue and Organ Culture* **20**: 47-55.

Artus, N. (2002). Effect of arsenate treatment on growth and arsenic accumulation in four high biomass members of the Brassicaceae. ***In***: The 9th New Phytologist Symposium. Biology Department, West Chester University, West Chester, USA. pp 193 – 195.

Ashraf, M. and Sharif R. (1998). Assessment of inter-cultivar variation for drought resistant in a potential oilseed crop Ethiopian mustard (*Brassica carinata* A. Braun). *Arch. Acker-Pfl. Boden.,* **43**: 251-265.

Askew, M. F. (2004). Agronomy guide. Generic guidelines on the agronomy of selected industrial crops. *Breeding* **123**: 587-589.

Attia, T., Busso C. and Röbbelen G. (1987). Digenomic triploids for an assessment of chromosome relationships in the cultivated diploid /*Brassica*/ species. *Genome* **29**: 326-330.

Babic, V., Datla, R. S., Scoles G. J. and Keller W. A. (1997). Marker assisted selection for *Brassica carinata Agrobacterium* mediated transformation *Plant Cell Rep.***17**: 183-188.

Bansal, K. C. (2002). Genetic engineering for enhancing abiotic stress tolerance: water use efficiency and nitrogen use efficiency. *National Research Center on Plant Biotechnology. Indian Agricultural Research Institute.* New Delhi.

Barro, F. and Martin A. (1999). Response of different genotypes of *Brassica carinata* to microspore culture. *Plant Breeding*, **118**: 79–81.

Barro, F., Fernandez-Escobar J., De la Vega M. and Martin A. (2001).Modification of glucosinolate and erucic acid contents in doubled haploid lines of *Brassica carinata* by Ultra Violet light treatment of isolated microspores. *Euphytica* **129**: 1–6.

Barro, F., J. Ferna´ ndez-Escobar, M. De la Vega, and A. Martin (2002). Modification of glucosinolate and erucic acid contents in doubled haploid lines of *Brassica carinata* by UV treatment of isolated microspores. *Euphytica* **129**:1-6.

Beranek, M., Bechynem M. and Klima M. (2007). Protoplast isolation and fusion between *Brassica carinata* A. Braun and *Brassica rapa* L. *Agricultura Tropica et Subtropica* **40**: 01 – 07.

Bhojwani, S. S., Sharma K. K. and Thorpe, T. A. (1990). Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Science* **66**:247–253.

Binarova, P., Hause G., Cenklova V., Cordewener J.H.G, and Van Lookern Campagne M.M. (1977). A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. *Plant Rep*. **10**: 200–208.

Bozzini, A., Calcagno, F. and Soare, T. (2007). "Sincron", a new *Brassica carinata* cultivar for biodiesel production. *HELIA*, **46**: 207-214.

Brown, P.D. and Morra M. J. (1997). Control of soil-borne plant pests using glucosinolate-containing plants. *Advances in Agronomy.* **61**: 167-231.

Bulcha Woyessa, Aduga Waqjira and Misteru Tesfaye (2007) . Highland Oil Crops Progress Report, Ethiopian Institute of Agricultural Research, Holetta Center.

Burbulis, N., Kupriene R. and Zilenaite L.(2004). Embryogenesis, callogenesis and plant regeneration from anther cultures of spring rape (*Brassica napus* L.). *Biology* **676**:153-158.

Busso, C., Attia T. and Röbbelen G. (1987). Trigenomic combinations for the analysis of meiotic control in the cultivated *Brassica* species. *Genome* **29**: 331-333.

Chen Y. (1986). Anther and pollen culture of rice. ***In***: Yang H. (ed) *Haploids of Higher Plants in vitro*. Springer, Berlin Heidelberg. New York. PP 3-25.

Chen Z.Z., Snyder S., Fan G.Z. and Loh W.H. (1994). Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. *Plant Breed.* **113**:217–221.

Choung P.V. Bevedrof W.D. (1985): High frequency embryogenesis through isolated microspore culture in *B. napus* and *B. carinata. Plant Science*. **39**: 219–226.

Chrungu B., Verma N., Mohanty A., Pradhan A. and Shivanna K. R. (1999). Production and characterization of interspecific hybrids between *Brassica maurorum* and crop *Brassica*s. *Theor. Appl. Genet.* **98**: 608-613.

Chu, C. C. (1978). The N6 medium and its application to anther culture of cereal crops. ***In***: *Proceedings on Symposium Plant Tissue Culture*, Science Press, Peking, pp. 43–50.

Courtny J. Murren and Massimo P. (2005). Morphological response to simulated wind in the genus Brassicaseae: allopolyploids and their parental species. *Amer. J. Bot.* **92**: 810–818.

CSA (Central Statistics Authority) (2009). Agricultural sample survey 2008/. Report on area and production for major crops. Statistical Bulletin 200, Addis Ababa. Ethiopia, 92 pp.

Cus t ers J. B. M., Cordewene r J. H. G., Dons H. J. M. and Lookeren M. M. V. (1996). Regulation of the inductive phase of microspore embryogenesis in *Brassica napus Acta Horticulturae* **407**: 209–217.

Datta, S. K., Datta, K. and Potrykus, I. (1990). Embryogenesis and plant regeneration from microspores of both Indica and Japonica rice (*Oryza sativa*). *Plant Sci*. **67**: 83–88.

Datta K. S. (2005). Androgenic haploids: Factors controlling development and its application in crop improvement. *Current Science* **89**: 1870-1878.

De Haro, A., Domınguez, J., Garcıa-Ruız, R., Velasco, L., Del Rıo, M., Munoz, J. and Fernandez-Martınez, J. (1998). Registration of six Ethiopian mustard germplasm lines. *Crop Science* **38**: 558 - 561.

Dunwell J. M., Cornish M. and De Courcel A.G.L. (1983). Induction and growth of "microspore-derived" embryo of *Brassica napus* ssp. *oleifera*. *J. Exp. Bot.* **34**: 1768–1778.

Edwards, S., Mesfin Tadesse, Sebsibe Demissew and Hedberg, I. (2000). *Flora of Ethiopia and Eriterea*. Macnoliaceae to Flacourtiaceae. National Herbarium, Addis Ababa, Ethiopia and Uppsala, Sweden, 121 - 125pp.

Efftoda S. (2002). Microspore Culture in *Brassica* species. *Plant Biotechnology*.**416**: 233-239.

Errie A.M.R. and Keller W.A. (1995). Microspore culture for haploid plant production. ***In***: Gamborg O.L., Phillips G.G. (eds.): *Plant Cell, Tissue and Organ Culture*: fundamental methods. Springer, Berlin, pp. 155–164.

Eyasu Abrha, Bechyne M., Klima M. and Vyadilova M. (2007). Embryogenic responsivity of selected genotypes of *Brassica carinata* A. Braun to microspore culture. *Agricultura Tropica et Subtropica*. **40**: 35-42.

Eyasu Abraha, Bechyn M., Klima M. and Vyvadilova M. (2008). Analysis of factors affecting embryogenesis in microspore cultures of *Brassica carinata*. *Agricultura Tropica et Subtropica.* **41**: 53-59.

Ferrie, A. M. R., Taylor, D. C., Mackenzie S. L., Rakow G., Raney J. P. and Keller W. A. (2008). Microspore mutagenesis of *Brassica* species for fatty acid modifications: a preliminary evaluation. *Plant Breeding*.**127**:501-506.

Food and Agricultural Organization of the United Nations (FAO) (2007). *B. carinata.* [ http//ecoport. org/ ep plant] 01, 29, 2007: 14: G. M. T.

Galli, L., Viegas, J., Augustin, E., Eckert, M. I. and Siva, J. B. (1998), Meiosis of anther culture regenerants in asparagus (*Asparagus officinalis* L.). *Genet. Mol. Biol*. **21**: 1415–4757.

Gamborg, O.L., Miller, R.A. and Ojima, K., (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*. **50**: 151-158.

Getinet Alemaw and Nigussie Alemayehu (1991). Production and Research in Oilseeds. **In**: *Proceeding of the First National Oilseeds Workshop, 3-5 December 1991*, pp. 31-38, IAR, Addis Ababa, Ethiopia.

Getinet Alemaw, Adefris Tekelewold, Mengistu Negi. and Tesfaye G.(1991). Rapeseed and gomenzer breeding in Ethopia **In**: *Proceeding of the First National Oilseeds Workshop, 3-5 December 1991*, pp. 31-38, IAR, Addis Ababa, Ethiopia

Getinet Alemaw, Rakow, G., Raney, J. P. and Downey, R. K. (1994). Development of zero erucic acid Ethiopian mustard through an interspecific cross with zero erucic acid Oriental mustard. *Can. J. Plant Sci*. **74**: 793-795.

Getinet Alemaw, Rakow G, and Downey R. K. (1996). Agronomic performance and seed quality of Ethiopian mustard in Saskatchewan. *Can J. Plant Sci*. **76**:387-392.

Gil-Humanes J. and Barro F. (2009). Production of doubled haploids in *Brassica*. **In**: Tournev *et al* (eds). *Advances in Haploid production in higher plants.* Cordoba. Spain, pp 65-70.

Gorecka K. and Krzyzanowska D. (1990). Plant regeneration from anther derived embryos of cabbage (*Brassica oleracea* L. Var. *captitata*). *Experimental Agriculture.* **26**:125-129.

González-García S., Gasol C.M., Gabarrell X., Rieradevall J., Moreira M.T. and Feijoo. G. (2009). "Environmental aspects of ethanol-based fuels from *Brassica carinata*: A case study of second generation ethanol". *Renewable and Sustainable Energy Reviews*, **13**: 2613-2620.

Gugel, R. K., Séguin-Swartz, G. and Petrie, A. (1990). Pathogenicity of three isolates of *Leptosphaeria maculans* on *Brassica* species and other crucifers. *Can. J. of Pl. Pat*. **12**: 75-82.

Guha, S. and Maheshwari, S. C. (1964), *In vitro* production of embryos from anthers of *Datura. Nature*. **204**: 497-501.

Gundimeda H. R., Prakash S. and Shivanna K. R. (1992). Intergeneric hybrids between *Enarthrocarpus lyratus,* a wild species, and crop *Brassica*s. *Theor. Appl. Genet*. **83**: 655-662.

Hemingway, J.S (1995). Mustards: *Brassica* spp. and *Snapsis alba* (Criciferae). **In**: *Evolution of Crop Plants.* 2nd ed Smart, J. and Simmonds, N. W. (eds). Longman Scientific and Technical, Singapore, 82 - 88 pp.

Henderson, C.A.P. and Pauls, K.P. (1992). The use of haploidy to develop plants that express several recessive traits using light-seeded canola (*Brassica napus*) as an example. *Theor. Appl. Genet*. **83**: 476-479.

Hiruy Belayneh (1985). Review of rape seed/ mustard breeding and agronomy research in Ethiopia. Paper presented at the workshop on review of field Crops Research in Ethiopia, Addis Ababa, Ethiopia, 25 Feb.-1 March, 1985.

Hiruy Belayneh and Nigussie Alemayehu (1986). Progress in rapeseed or mustard research in Ethiopia. **In**: Omran *Proceeding of The Third Oil Crops Network Workshop.IDRCMR,153e 3-5 December 1991*, pp. 31-38, IAR, Addis Ababa, Ethiopia. IDRC Ottawa, Canada.

Huang B., Birds S., Kemble R., Simmonds D., Keller W.A. and Miki B. (1990). Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. Topas. *Plant Cell Reports*. **8**: 594–597.

Jane A. and Lorz H. (1995). Cereal microspore culture. *Plant Science* .**109**: 1-12.

Keller W. A. and Armstrong K. C. (1977). Embryogenesis and plant regeneration in *Brassica napus* anther culture. *Can. J. Bot*. **55**: 1383-1388.

Keller W.A., Armstrong K.C. (1979): Stimulation of embryogenesis and haploid production in *Brassica rapa* anther cultures by elevated temperature treatments. *Theor. Appl. Gen*et, **55**: 65– 67.

Kim M., Kim J., Yoon M., Choi D. I. and Lee K. M. (2004). Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annum*). *Plant Cell Tissue Organ culture* **77**: 63-72.

Kott L.S. and Beversdorf W.D. (1988): Cytological aspects of isolated microspore culture in *Brassica napus*. *Can. J. Bot*. **66**: 1665–1670.

Kott L.S. and Beversdorf W.D. (1990). Enhanced plant regeneration from microspore derived embryos of *Brassica napus* by chilling, partial desiccation and age selection. *Plant Cell Tissue Organ Culture* **23**: 187–192.

Kott L.S. (1998). Application of doubled haploid technology in breeding of oilseed *Brassica napus*. *Plant Cell Tissue Organ Culture* **23**: 187–192.

Krishnan, G., Holshouser, D. L. and Nissen, S. J. (1998). Weed control in soybean (*Glycine max*) with green manure crops. *Weed Technology*. **12**: 97-102.

Lazzeri, L. and Avino D. L. (2009). Green Chemistry: an agricultural production chain for biolubricants. **In**: workshop on Green Chemistry Future and its Possible Impact on Agriculture, the Whole Farm Tractability Approach . Ispara , Italy.

Liang Z. S., Ding Z. R. and Wang S. T. R. (1992). Study on type of water stress adaptation in both *Brassica napus* and *B. juncea* L. species. *Acta Botanika.* **12**: 38–45.

Lichter R (1989). Efficient yield of embryoids by culture of isolated microspores of different Brassicacae species. *Plant Breeding.* **103**: 119-123.

Lichter R. (1982). Induction of haploids plants from isolated pollen of *Brassica napus*. *Z. pflanzenphysiol*. **105**: 427–434.

Loh C. S. and Ingram D. S. (1983). Production of haploid plants from anther cultures and secondary embryoids of winter oilseed rape *Brassica napus* ssp. *oleifera. New Phtyol.* **91**:507-516.

Macdonald, M.V., Hadwiger, M. A., Aslam F. N. and Ingram D. S. (1988).The enhancement of anther culture efficiency in *Brassica napus* ssp. oleifera Metzg.(Sinsk.)using low doses of gamma irradiation. *New Phytol*.**110**:101-107.

Macdonals M. V. and Ingram D. S. (1984). The use of tissue culture in oilseed rape breeding. ***In***: Aspects of Applied Biology, Agronomy, Physiology, Plant Breeding and Crop

Protection of Oilseed Rape. Association of Applied Biologists, Wellesbourne. pp 37-44.

Malik R.S. (1990): Prospects for *Brassica carinata* as an oilseed crop in India. *Exp.Agric*. **26**: 125-129.

Meng J. L., Shi S. W., Li G. and Li Z. Y. (1998). The production of yellow-seeded *B. napus* (AACC) through crossing interspecific hybrids of *B. campestri*s (AA) and *B. carinata* (BBCC) with *B. napus*. *Euphytica*. **103**: 329-333.

Miao Y. , Ding Y. U., Qiao-YangSun, Zeng- Fu X. U. and Liwen J.(2008). Plant bioreactors for pharmaceuticals. *Biotechnology and Genetic Engineering*. **25**: 363-380.

Moffat A. S. (2002). New ways to protect drought-stricken plants. *Science* **296**:1226-1229.

Momotaz A., Kato M. and Kakihara F. (1998). Production of intergeneric hybrids between *Brassica* and *Sinapis* species by means of embryo rescue techniques. *Euphytica*. **103**: 123-130.

Moon, H. P. (1998). An anther-derived, early-maturing and cold-tolerant rice variety 'Hwadongbyeo'. *Kor. J. Breeding*. **30**:382 - 386.

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15**: 473-479.

Muthoni J. (2010). Characterization of Ethiopian mustard (*Brassica carinata* A. Braun) lines for vegetative agromorphological traits at Arusha, Tanzania. *Journal of Horticulture and Forestry.* **2**: 001-006.

Nitsch, J.P. and Nitsch, C. (1969). Haploid plants from pollen grains. *Science*, **163**: 85- 87.

Nigussie Alemayhu, Adefris Teklewold and Tadele Zerihun (1996). Effect of agronomic practices on seed and oil yields of Ethiopian mustard (*Brassica carinata* A. Braun and rapeseed (*B. napus* L.). *Trop. Agric*. **73**:94-99.

Negussie Alemayehu and Becker H.C. (2001). Variation and inheritance of erucic acid content in *Brassica carinata* germplasm collections from Ethiopia. *Plant Breeding*. **120**:331-335.

Negussie Alemayehu and Becker, H.C. (2002). Genotypic diversity and pattern of variation in a germplasm material of Ethiopian mustard (*Brassica carinata* A. Braun). *Genet Resour. Crop Evol.* **49**: 573 - 582

Oleszek, W. (1987). Allelopathic effects of volatiles from some cruciferae species on lettuce, barnyard grass and wheat growth. *Plant and Soil.* **102**: 271-273.

Olsen, F. L. (1988). Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*. The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. *Carlsberg Res. Commun*. **52**:393-404.

Pechan P.M. and Keller W.A. (1988). Identification of potentially embryogenic microspores in *Brassica napus*. *Physiol. Plant*. **74**: 377–384.

Phelps S. (2008). *Oilseeds for biofuel production in Scott Field*. Saskatchewan, Canada

Rakow G., Getinet Alemaw, Raney J. P. and Downey R. K. (1994). Development of zero erucic acid Ethiopian mustard through an interspecfic cross with zero erucic acid Oriental mustard. *Can. J. Plant Sci.* **74**:793-795.

Rakow, G. (1995). Developments in the breeding of edible oil in other *Brassica* species. ***In***: Proceedings of the 9th Int. Rapeseed Conf., Cambridge, Henry Ling Ltd. Dochester, UK 410 - 412 pp.

Rakow G. (2004). Species origin and economic importance of *Brassica. Biotechnology in Agriculture and Forestry*. **54**: 3-7.

Rao G. U., Lakshmi kumaran M. and Shivanna K. R. (1996). Production of hybrids, amphiploids and backcross progenies between a cold-tolerant wild species, *Erucastrum abyssinicum* and crop *Brassica*s. *Theor. Appl. Genet.* **92**: 786-790.

Roy A. and Saha P. K. (2006). Isolation of low erucic acid-containing genotype of Indian mustard (*Brassica juncea* Czern. and Coss.) through $F_1$ hybrid anther culture. *African Journal of Biotechnology.* **22**: 2092-2096.

Sabharwal P. S. and Dolezel J. (1993). Interspecific hybridization in *Brassica*: application of flow cytometry for analysis of ploidy and genome composition in hybrid plants. *Biologia Plantarum.* **35**: 169-177.

Sacristán M. D. and Gerdemann M.(1986). Different behavior of *B. juncea* and *B. carinata* as sources of *Phoma lingam* resistance in experiments of interspecific transfer to *B. napus*. *Plant Breeding*. **97**: 304-314.

Schippers, R.R. (2002). An overview of the cultivated African Indigenous vegetables. *Science*.**296**:1226-1229.

Setia, R.C., and Richa. (1989). Anatomical studies on siliquae wall and seed coat development in *Brassica juncea* L Czern and coss. *Phytomonphology* **39**: 371-377.

Sharma, K. K., Bhojwani, S. S. and Thorpe, T. A. (1990). Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Sci*. **66**:247–253.

Sharma T. R. and Singh B. M. (1992). Transfer of resistance to *Alternaria brassicae* in *Brassica juncea* through interspecific hybridization among *Brassica*s. *Journal of Genetics and Breeding.* **46**: 373-378.

Simmonds, N.W. (1979). *Longman Principles of Crop Improvement Group*, New York.

Singh, D. (2003). Genetic improvement in Ethiopian mustard (*Brassica carinata* A. Braun). vis a vis Indian mustard (*Brassica juncea* L. Czern and Coss.). **In**: Proc.11$^{th}$ Int. Rapeseed Confr. Copenhagen, Denmark, 513pp.

Singh, D. and Singh, H. (1988). Inheritance of white rust resistance in interspecific crosses of *Brassica juncea L.* and *B. carinata L. Crop Resources* (Hisar). **1**: 189-193.

Smyykalova I., Martina V., Miroslav K., Ivana M. and Miroslav G. (2006). Efficiency of microspore culture for doubled haploid production in the breeding project "Czech Winter Rape" Czech. *J. Genet. Plant Breed.* **42**: 58–71.

Sopory S. K. and Munchi M. (1996). Anther culture. *In Vitro Haploid Production in Higher Plants* (eds) Monhanjain S. M., Sopory S. K. and Veilleux, R. E. Kluwer Academic Publishers, Dordrecth, pp 145-176.

Sorvarsi S. and Schieder O. (1987). Influence of sucrose and melibiose of barley anther cultures in starch media. *Plant Breed.* **99**: 164-171.

Sridevi O. and Sarla N. (1996). Reciprocal hybridization between *Sinapis alba* and *Brassica* species. *Cruciferae Newsletter.* **18**: 16-24.

Steinmetz, K. A. and Potter, J. D. (1996).Vegetables, fruits and cancer prevention: A review. *J. Am. Diet. Assoc*. **96**:1027–1039.

Stymne S. (2008). *Genetic Engineered Vegetable Oils for Bio-materials and Bio-fuels*. Swedish University of Agricultural Science. Sweden, Stockholm.pp.87-92.

Sunderland, N. and Dunwell, J. M. (1974). Pathways in pollen embryogenesis. *Tissue Culture and Plant Science.* **27**: 141–147.

Swapan, K.D. (2005). Androgenic haploids: Factors controlling development and its application in crop improvement. *Current Science*. **89**: 1870-1878.

Telmer C.A., Simmonds D.H. and Newcomb W. (1992). Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. *Physiol. Plant* **84**: 47–424.

Telmer, C. A., Newcomb W. and Simmonds D. H. (1993). Microspore development in *Brassica napus* and the effect of high temperature on division *in vivo* and *in vitro*. *Protoplasma.* **172**: 154-1 65.

Thurling N. and Chay P.M. (1984). The influence of donor plant genotype and environment on production of multicellular microspores in culture anthers of *Brassica napus* spp. Oleifera. *Annals of Botany*. **54**:681–695.

Tonguç M. and Griffiths P. D. (2004). Transfer of powdery mildew resistance from *Brassica carinata* to *Brassica oleracea* through embryo rescue. *Plant Breeding.* **123**: 587-589.

Touraev A, Vicente O, Heberle-Bors E. (1997). Initiation of microspore embryogenesis by stress. *Trends.Plant Sci*. **2**: 297-302.

U, N. (1935). Genome analysis in *Brassica* with specific reference to the experimental formation of *B. napus* and peculiar mode of fertilization. *Japanese J. Bot.* **7**: 389 – 452.

Velasco, L., Goffman, F.D. and Becker, H.C. (1998). Variability for the fatty acid composition of the seed oil on germplasm collection of the genus *Brassica*. *Genet. Resour.Crop. Evol*. **45**: 371 - 382.

Velasco, L., Nabloussi, A., Haro, A. D. and Fernandez-martinez, J. (2004). Allelic variation in linoleinic acid content of high erucic acid Ethiopian mustard and incorporation of the low linoleinic acid trait into zero erucic acid germplasm. *Plant Breed.* **123**: 137 - 143.

Vera, C. L., McGregor, D. I. and Downey, R. K. (1987). Detrimental effects of volunteer Brassica on production of certain cereal and oilseed crops. *Can. J. Plant Sci*. **67**: 983-995.

Vincente J.G., Dias J.S. (1996). Production of embryos from microspore cultures of Portugese tronchuda cabbage landraces. In: Dias J.S., Crute I., Monteiro A.A.: *International*

*Symposium on Brassicas Ninth Crucifer Genetics Workshop*. *ACTA Horticulturae*, **407**. ISHS, pp. 219–226.

Wang M., Farnham, W. and Mannes, J. S. P. (1999). Ploidy of broccoli regenerated from microspore cultures versus anther culture. *Plant Breed*. **118**:249–252.

Warwick, S. I., Gugel, R. K., McDoald, T. and Falk, K. C. (2006). Genetic variation of Ethiopian mustard (*Brassica carinata* A. Braun) germplasm in western Canada. *Gen. Res. Cro. Evo*. **53**: 297-312.

Wong P.T.W., Sarwar M., Kirkegaard J.A. and Desmarchelier J.M., (1998). Biofumigation potential of *Brassica*s .III. *In vitro* toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil*. **201**: 103-112.

Yitbarek Semeane (1992). Pathological research on noug, linseed, gomenzer and rapeseed in Ethiopia. **In**: *Oilseeds Research and Development in Ethiopia, Proceedings of the First National Oilseeds Workshop*, 3 - 5 December 1991, Addis Ababa, Ethiopia 151 - 161 pp.

Zaki, M. and. Dickinson. H. G (1990). Structural changes during the first division of embryos from anther and microspore culture in *Brassica napus*. *Protoplasma.* **156**: 149- 162.

Zemede Asfaw. (1995). Conservation and use of traditional vegetables in Ethiopia. **In**: *Proceedings of IPGRI Workshop on Genetic Resources of Traditional \Vegetables in Africa*, *Conservation and Use.* Guarino, L. (ed) 29-31 August 1995, TCRAF-HQ, Nairobi, Kenya. 57 - 63 pp.

Zhao J., Newcomb W. and Simmonds D. (2003). Heat-Shock Proteins 70 kDa and 19 kDa are not Required for Induction of Embryogenesis of *Brassica napus* L. cv. Topas microspores. *Plant Cell Physiol.* **44**: 1417–1421.

## APPENDIX I. The four (Ms, NN, B5 and N6) basal media components and concentrations

Table 1. Murashige and Scoog (MS) basal medium nutrient composition and concentration

| | Component | Concentration (mg/l) |
|---|---|---|
| **Macro** | $CaCl_2.2H_2O$ | 332.2 |
| | $KH_2PO_4$ | 170 |
| | $KNO_3$ | 1900 |
| | $MgSO_4$ | 180.7 |
| | $NH_4NO_3$ | 1650 |
| **Micro** | $CoCl_2.6H_2O$ | 0.025 |
| | $CuSO_4.5H_2O$ | 0.025 |
| | $FeSO_4$ | 27.8 |
| | $H3BO_3$ | 6.2 |
| | KI | 0.83 |
| | $MnSO_4.H_2O$ | 1.69 |
| | $NaMoH_2$ | 0.25 |
| | NaEDTA | 37.26 |
| | $ZnSO_4.7H_2O$ | 8.6 |
| **Organic Supplements** | Glycine | 2.0 |
| | Myo-inositol | 100 |
| | Nicotnic acid | 0.5 |
| | Pyridoxin (HCl) | 0.5 |
| | Thiamin (HCl) | 0.1 |

Table 2. Gamborge($B_5$) basal medium nutrient composition and concentration

| | Component | Concentration (mg/l) |
|---|---|---|
| **Macro** | $CaCl_2.2H_2O$ | 332.2 |
| | $KNO_3$ | 2500 |
| | $MgSO_4$ | 122.09 |
| | $NaHPO_4$. Monobasic | 130.5 |
| | $NH_4SO_4$ | 134.0 |
| **Micro** | $CoCl_2.6H_2O$ | 0.025 |
| | $CuSO_4.5H_2O$ | 0.025 |
| | $FeSO_4$ | 27.8 |
| | $H_3BO_3$ | 6.2 |
| | KI | 0.75 |
| | $MnSO_4.H_2O$ | 10 |
| | NaEDTA | 37.3 |
| | $NaMoO_4.2H_2O$ | 0.25 |
| | $ZnSO_4.7H_2O$ | 2 |
| **Organic Supplements** | Myo-inositol | 100 |
| | Nicotnic acid | 1.0 |
| | Pyridoxin HCl | 1.0 |
| | Thiamin HCl | 10 |

Table 3. Nitches-Nitcthes (NN)basal medium nutrient composition and concentration

| | **Component** | **Concentration (mg/l)** |
|---|---|---|
| **Macro** | $CaCl_2.2H_2O$ | 166 |
| | $KH_2PO_4$ | 68 |
| | $KNO_3$ | 950 |
| | $MgSO_4$ | 185 |
| **Micro** | $CoCl_2.6H_2O$ | 0.025 |
| | $CuSO_4.5H_2O$ | 0.025 |
| | $FeSO_4$ | 27.8 |
| | $H_3BO_3$ | 10 |
| | KI | 0.83 |
| | $MnSO_4.H_2O$ | 25 |
| | $NaMoO_4.2H_2O$ | 0.25 |
| | NaEDTA | 37.3 |
| | $ZnSO_4.7H_2O$ | 10 |
| **Organic Supplements** | Biotin | 0.2 |
| | Glycine | 2.0 |
| | Myo-inositol | 100.0 |
| | Nicotnic acid | 5.0 |
| | Pyridoxin ($B_6$) | 0.5 |
| | Thiamin ($B_1$) | 0.5 |

Table 4. Chu ($N_6$) basal medium nutrient composition and concentration

| | **Component** | **Concentration (mg/l)** |
|---|---|---|
| **Macro** | $CaCl_2.2H_2O$ | 125.33 |
| | $KNO_3$ | 2830.0 |
| | $MgSO_4$ | 90.37 |
| | $KHPO_4$. Monobasic | 400.0 |
| | $NH_4SO_4$ | 463.0 |
| **Micro** | $FeSO_4$ | 27.85 |
| | $H_3BO_3$ | 1.6 |
| | KI | 0.8 |
| | $MnSO_4.H_2O$ | 3.33 |
| | NaEDTA | 37.25 |
| | $ZnSO_4.7H_2O$ | 1.5 |
| **Organic Supplements** | Glycine (free base) | 2.0 |
| | Nicotnic acid | 0.5 |
| | Pyridoxin HCl | 0.5 |
| | Thiamin HCl | 1.0 |

**APPENDIX II.** Tables showing effects of bud stage difference, basal media and incubation duration on embryo induction, plantlet regeneration and root development

**Table 1. The effect of bud stage difference on embryo induction**

| Gen | Rep | $B_1$ | $B_2$ | $B_3$ |
|---|---|---|---|---|
| Holeta 1 | 1 | 24 | 6 | 1 |
| | 2 | 27 | 3 | 1 |
| | 3 | 21 | 2 | 0 |
| mean | | **24** | **3.66** | **0.66** |
| YD | 1 | 18 | 0 | 0 |
| | 2 | 23 | 5 | 2 |
| | 3 | 14 | 1 | 0 |
| mean | | **18.33** | **2** | **0.66** |
| S-67 | 1 | 15 | 8 | 0 |
| | 2 | 22 | 7 | 0 |
| | 3 | 20 | 5 | 4 |
| mean | | **19** | **6.66** | **1.33** |
| PGRCE07/1/2 | 1 | 31 | 3 | 3 |
| | 2 | 19 | 0 | 0 |
| | 3 | 26 | 8 | 1 |
| mean | | **25.33** | **3.66** | **1.33** |
| Holeta 1 | 1 | 24 | 6 | 1 |
| | 2 | 27 | 3 | 1 |
| | 3 | 21 | 2 | 0 |
| mean | | **24** | **3.66** | **0.66** |
| YD | 1 | 18 | 0 | 0 |
| | 2 | 23 | 5 | 2 |
| | 3 | 14 | 1 | 0 |
| mean | | **18.33** | **2** | **0.66** |
| S-67 | 1 | 15 | 8 | 0 |
| | 2 | 22 | 7 | 0 |
| | 3 | 20 | 5 | 4 |
| mean | | **19** | **6.66** | **1.33** |
| PGRCE07/1/2 | 1 | 31 | 3 | 3 |
| | 2 | 19 | 0 | 0 |
| | 3 | 26 | 8 | 1 |
| mean | | **25.33** | **3.66** | **1.33** |

**Table 2. Data for the effect of basal media difference on embryo induction**

| Variety | Medium | $R_1$ | $R_2$ | $R_3$ | Mean |
|---|---|---|---|---|---|
| Holeta 1 | NN | 42 | 39 | 40 | **40.3** |
| Holeta 1 | $B_5$ | 38 | 34 | 37 | **36.3** |
| Holeta 1 | MS | 19 | 21 | 17 | **19** |
| Holeta 1 | $N_6$ | 4 | 6 | 3 | **4.3** |
| Yellow Do | NN | 15 | 13 | 17 | **15** |
| Yellow Do | $B_5$ | 41 | 37 | 44 | **40.66** |
| Yellow Do | MS | 13 | 15 | 16 | **14.66** |
| Yellow Do | $N_6$ | 0 | 1 | 2 | **1** |
| S-67 | NN | 8 | 10 | 7 | **8.33** |
| S-67 | $B_5$ | 26 | 24 | 28 | **26** |
| S-67 | MS | 10 | 7 | 14 | **10.3** |
| S-67 | $N_6$ | 4 | 3 | 6 | **4.33** |
| PGRCE07/1/2 | NN | 2 | 4 | 1 | **2.33** |
| PGRCE07/1/2 | $B_5$ | 42 | 44 | 38 | **41.33** |
| PGRCE07/1/2 | MS | 24 | 18 | 22 | **21.33** |
| PGRCE07/1/2 | $N_6$ | 2 | 1 | 3 | **2** |

**N. B.**

**$R_1$=** Replication 1

**$R_2$=** replication 2

**$R_3$=** Replication3

**Table 3. The effect of temperature incubation duration difference on embryo induction**

| Medium | Gen | H0 | H1 | H2 | H3 |
|---|---|---|---|---|---|
| NN | Holeta 1 | 1 | 28 | 10 | 3 |
| NN | Yellow Do | 2 | 7 | 5 | 3 |
| NN | S-67 | 0 | 4 | 1 | 3 |
| NN | PGRCE07/1/2 | 0 | 2 | 0 | 0 |
| $B_5$ | Holeta 1 | 3 | 21 | 10 | 4 |
| $B_5$ | Yellow Do | 3 | 34 | 4 | 0 |
| $B_5$ | S-67 | 4 | 16 | 6 | 0 |
| $B_5$ | PGRCE07/1/2 | 2 | 40 | 0 | 0 |
| MS | Holeta 1 | 0 | 19 | 0 | 0 |
| MS | Yellow Do | 0 | 11 | 1 | 1 |
| MS | S-67 | 4 | 6 | 0 | 1 |
| MS | PGRCE07/1/2 | 5 | 17 | 2 | 0 |
| $N_6$ | Holeta 1 | 0 | 3 | 1 | 0 |
| $N_6$ | Yellow Do | 0 | 0 | 0 | 0 |
| $N_6$ | S-67 | 1 | 1 | 2 | 0 |
| $N_6$ | PGRCE07/1/2 | 0 | 2 | 0 | 0 |

**H0=** Control for Incubation duration

**H1=** Incubation duration 1

**H2=** Incubation duration 2

**H3=** Incubation duration 3

**Table 4. Data for embryo - plantlet regeneration**

| Variety | Media | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Mean |
|---|---|---|---|---|---|---|---|
| Holeta 1 | Trt 0 | 32 | 27 | 25 | 36 | 19 | **27.8** |
| Holeta 1 | Trt 1 | 0 | 1 | 0 | 0 | 2 | **0.6** |
| Holeta 1 | Trt 2 | 2 | 0 | 0 | 0 | 1 | **0.6** |
| Holeta 1 | Trt 3 | 1 | 2 | 0 | 0 | 3 | **1.2** |
| Yellow Do | Trt 0 | 24 | 28 | 33 | 29 | 40 | **30.8** |
| Yellow Do | Trt 1 | 0 | 1 | 1 | 0 | 0 | **0.4** |
| Yellow Do | Trt 2 | 2 | 1 | 1 | 0 | 3 | **1.4** |
| Yellow Do | Trt 3 | 1 | 0 | 0 | 0 | 1 | **0.4** |
| S-67 | Trt 0 | 32 | 21 | 19 | 37 | 29 | **27.6** |
| S-67 | Trt 1 | 0 | 0 | 0 | 1 | 1 | **0.4** |
| S-67 | Trt 2 | 0 | 2 | 2 | 1 | 0 | **1** |
| S-67 | Trt 3 | 0 | 0 | 3 | 1 | 0 | **0.8** |
| PGRCE07/1/2 | Trt 0 | 29 | 41 | 36 | 26 | 38 | **34** |
| PGRCE07/1/2 | Trt 1 | 0 | 0 | 1 | 1 | 1 | **0.6** |
| PGRCE07/1/2 | Trt 2 | 0 | 0 | 0 | 2 | 0 | **0.4** |
| PGRCE07/1/2 | Trt 3 | 2 | 3 | 3 | 1 | 1 | **2** |

**$R_1$=** Replication 1

**$R_2$=** Replication 2

**$R_3$=**Replication 3

**$R_4$=** Replication 4

**$R_5$=** Replication 5

**DECLARATION**

I the undersigned, declare that this thesis is my original work and has not been presented for s degree in any other University. All sources of materials used for the thesis have been duly acknowledged.

---

Yemisrach Zewdu Belay

This thesis has been submitted for examination with my approval as a University advisor.

---

Tileye Feyissa (phD)