FACTORS AFFECTING *IN VITRO* CALLUS GROWTH AND REGENERATION OF FLAX (*Linum usitatissimum* L.)

By

ZEINAB KASSEM TAHA SHARAWY
B.Sc. Agric. Sci. (Biotechnology), Fac. Agric., Cairo Univ., 2003

THESIS
Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

In

Agricultural Sciences
(Agricultural Botany)

Department of Agricultural Botany
Faculty of Agriculture
Cairo University
EGYPT

2010

APPROVAL SHEET

FACTORS AFFECTING *IN VITRO* CALLUS GROWTH AND REGENERATION OF FLAX (*Linum usitatissimum* L.)

M.Sc. Thesis
In
Agric. Sci. (Agricultural Botany)

By

ZEINAB KASSEM TAHA SHARAWY

B.Sc. Agric. Sci. (Biotechnology), Fac. Agric., Cairo Univ., 2003

APPROVAL COMMITTEE

Dr. HUSSEIN SAYED TAHA.....
Researcher Professor of Plant Bio -Technology, National Research Center

Dr. MOKHTAR HASSAN ABDEL –RAHMAN ABU – BAKR.....
Professor of Agricultural Botany, Fac. Agric., Cairo University

Dr. ABD EL-HAMID ALI MOHAMED.....
Emeritus Professor of Agricultural Botany, Fac. Agric., Cairo University

Date: / /

SUPERVISION SHEET

FACTORS AFFECTING *IN VITRO* CALLUS GROWTH AND REGENERATION OF FLAX (*Linum usitatissimum* L.)

M.Sc. Thesis
In
Agric. Sci. (Agricultural Botany)

By

ZEINAB KASSEM TAHA SHARAWY
B.Sc. Agric. Sci. (Biotechnology), Fac. Agric., Cairo Univ., 2003

SUPERVISION COMMITTEE

Dr. ABD EL-HAMID ALI MOHAMED
Emeritus Professor of Agricultural Botany, Fac. Agric., Cairo University

Dr. ATEF ZAKARIA SABH
Professor of Agricultural Botany, Fac. Agric., Cairo University

Name of Candidate: Zeinab Kassem Taha Sharawy **Degree:** M.Sc.
Title of Thesis: Factors affecting *in vitro* callus growth and regeneration of flax (*Linum usitatissimum* L.)
Supervisors: Dr. Abd El-Hamid Ali Mohamed
Dr. Atef Zakaria Sabh
Department: Agricultural Botany
Branch: Economical Botany **Approval: / /2010**

ABSTRACT

Factors affecting *in vitro* callus induction, callus growth, shoot and root regeneration of two flax cultivars Giza 8 and Blanka were studied. Results revealed that hybocotyl explants of 3 days aged when cultured on MS medium supplemented with 0.5 mg / l NAA + 0.25 mg/ l BA proved to be the most suitable conditions for callus culture. The most inductive variant for direct shoot regeneration was the use of hybocotyl explants cultured on MS medium supplemented with 0.25 mg / l NAA + 0.50 mg / l BA. Scanning microscopy revealed the superficial initiation of shoot bud primordia. Comparative anatomy of *in vitro* versus *in vivo* shoots showed differences in epidermal, cortical and vascular tissues. For rooting 0.5 MS basal medium + 2g / l activated charcoal was the most effective. Callus cells were subjected to salinity stress (5 Na Cl: 1 Ca Cl_2) at levels up to 16000 ppm, growth rate and fresh weight were progressively reduced. Callus of both cultivars tolerated salinity stress ($NaCl + CaCl_2$) up to 8000 ppm. Salinity and enhanced the accumulation of Na, Cl, P, total soluble and reduced sugars and proline while levels of k was decreased with elevating salinity stress. Salinity and heavy metals ($PbCl_2$ and $CdCl_2$) inhibited both indirect and direct shoot regeneration. The used protocols achieved considerable shoot regeneration under salinity stress up to 8000 ppm and heavy metals stress; cadmium and lead chlorides up to 200 µM. The possibility of using tissue culture technique for selection of stress tolerant plants was discussed.

Key words: Adventitious Bud, BA, Flax, *Linum usitatissimum* L., Shoot Regeneration, NAA.

DEDICATION

I dedicate this work to whom my heart felt thanks; to Allah in the first my parents, my daughters, my husband, my sisters and my brothers and for all the support they lovely offered along the period of my post graduation.

ACKNOWLEDGEMENT

I wish to express my sincere thanks, deepest gratitude and appreciation to Dr. ***Abd El-hamid ALI Mohamed*** *and Dr.* ***Atef Zakaria Sabh*** *Professors of Agricultural Botany, Faculty of Agriculture, Cairo University for suggesting the problem, supervision, continued assistance and their guidance through the course of study and revision the manuscript of this thesis. Sincere thanks to Dr.* ***Ali Mahmod Saboor*** *Professor of Botany, Faculty of Agriculture; Cairo University. Sincere thanks to Dr.* ***Monir Mohamed El Hoseny*** *Professor of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University and Sincere thanks to Dr.* ***Gamal Amin Ghanem*** *Professor of Plant Pathology, Faculty of Agriculture, Cairo University*

Grateful appreciation is also extended to all staff members of Agriculture Botany Department, Faculty of Agriculture; Cairo University.

CONTENTS

INTRODUCTION

Flax is a dicotyledonous plant (*Linum usitatissimum* L.) from linaceae family and a very important crop for its high quality oil and fiber. Flax is a crop species widely an adapted to warm and cool temperate climates (Green, 1986) .In addition, it is one of the most earliest domestic and cultivated plant species. The genus *Linum* contains about 230 species, *Linum usitatissimum* is the only species which fulfills a commercial function, serving as a fiber donor for the textile industry and the production of linseed oil, (Magdalena *et al*. 2005), for the preparation of paints, varnishes, inks, linoleum and pharamaceaticals. (Green and Marshall, 1984, Belonogova and Raldugina, 2006 and Yildiz and Ozgen, 2006).

The linseed oil contains high amount of short- chain omega-3-fatty acids. In addition flax seeds contain high levels of dietary fiber including lignans and abundance of micronutrients. Lignans are good antioxidants they have antibacterial, antiviral properties and help prevent free-radical damage associated with aging and disease. Lignans protect the cardiovascular system by lowering LDL cholesterol.

Flax fiber is extracted from the bast of the stem, flax fiber is soft, lustrous and flexible, it is stronger than cotton fiber but less elastic. The best grades are used for linen fabrics. Flax fiber is also a raus material for the high quality paper industry for the use of printed banknotes and tea bags.

For the last 15 years, there has been a steady growing interest in the medicinal and nutraceutical value of flax, including experimental

evidence for its use in the prevention of cancer and cardiovascular and kidney diseases.

Plant tissue culture technique is widely used in plant propagation through organogenesis or somatic embryogenesis and is an essential tool in the application of molecular genetics to crop improvement. Callus development is readily obtained from many species but the regeneration of new plants, either from callus or directly from the original explants, is more problematic (Hazarika, 2006). Flax various explant sources, such as hypocotyls, Cotyledon, leaf, protoplast and anthers have been used for regeneration of flax. However no standard protocol for high efficient *in vitro* regeneration of flax has been yet adopted (Blinstrubiene *et al*., 2004).

In vitro flax regeneration through direct organogenesis is the most valuable method as having higher regeneration percentage and lower number of somaclonal variants in comparison with regeneration through embryogenesis (Dedicova *et al.*, 2000) or indirect organogenesis (Bretagne *et al*., 1994). For shoot regeneration, various flax explants were used; hypocotyl (Lane, 1979), cotyledon (Belonogova and Raldugina 2006), root (Cristina *et al*., 1997), anther (Burbulis *et al.* 2007), ovary (Bartosova 2006), protoplast (Ling and Binding, 2006). On the other hand, (Blinstrubiene *et al*., 2004) reported that flax shoot regeneration largely depends on the genotype, while (Cristina *et al*., 1997), stated a genotype- independent procedure. Although flax shown to be responsive *in vitro* to a wide range of growth regulators including thidiazuron (Bretagne *et al*., 1994), Naphthalene Acetic Acid and Benzyl Adenine are the most commonly

used for direct shoot regeneration. Flax hypocotyl segments from 5 and 7 day old seedlings were cultivated *in vitro* for direct regeneration by Dedicova *et al*. (2000) and Yildiz and Ozgen (2006).

There are very few reports on the effect of salinity and heavy metals on flax cells *in vitro*; Mc Hughen and Swartz (1984) and Charkavarty and Srivastava (1997).

Although this field of research is very important for production of new flax lines tolerant to different type of stress, such lines could be used not only for the economic production of flax crop but also for the phytoremediation of polluted soils.

The objectives of this study were to

1. Evaluate two cultivars of flax in relation to their ability to produce callus from explants of different organs under different conditions of cultivation *in vitro.*
2. Study the morphology and growth behavior of produced calli.
3. Study the regeneration capacity of different types of explants (direct regeneration).
4. Study the anatomical aspects of shoot buds regeneration.
5. Study the differential aspects of anatomical structure of various organs produced via *in vitro* and *in vivo* cultivation.
6. Study the growth kinetics, chemical components and regeneration capacity of callus cells under stress condition; salinity ($NaCl + CaCl_2$) and heavy metals (Pb and Cd).

7. Study the possibility of using tissue culture techniques in producing new lines of flax tolerant to stress condition.

REVIEW OF LTERATURE

1. Callus production

James *et al.* (1952) described a simple technique for the *in vitro* production of sunflower stem callus. This technique includes using of MS medium containing mixture of auxin (IAA), adenine, vitamins, and casein hydrolysate. This medium was found to support successfully the continued growth of callus tissue. Also, they reported that the addition of coconut milk to the nutrient medium greatly accelerates the growth of normal sunflower callus. Coconut milk and the supplement mixture together to MS medium gave significantly greater growth than any other combination.

Maria *et al.* (1987) found that, callus was induced from *in vitro* hypocotyl and cotyledon explants of *Helianthus annuus* L. var. *Argentario* on MS medium containing 2 mg/l NAA and 0.5 mg/l BA.

Gareth *et al*. (1996) cultured anthers of eight genotypes of linseeds on solid and in liquid medium. They stated that callus induction rate was low in both media and also the cultivars significantly affected on callus induction rate on solid and in liquid medium. The cultivar Blue-Chip gave the maximum of callus induction rate (3.67%) on solid medium and the cultivar McGregor gave the maximum of callus induction rate (3.35%) in liquid medium. Moreover, the cultivar Norman produced callus on solid medium but did not produce any callus in liquid medium. On the other hand, the cultivar McGregor did not produce any callus in either solid or liquid

medium. Pre-treatment of flower buds with cold significantly reduced callus induction rate by about four fold compared with the no pre-treatment on MS medium solid but increased callus induction rate by 25.8% in liquid medium.

Gomes and Fernandes (1996) mentioned that using of 2, 4-D and zeatin were the most efficient PGR combination tested for green callus induction from different explants of flax. The obtained results revealed that the callus induction can be manipulated on flax explants by raising the 2, 4-D level from 0.05 to 3.2 mg/l in the induction medium.

Perumal *et al.* (1996) applied different concentrations and combination of auxins and cytokinins for affecting callus induction from various explants of *Arachis hypogagaea* L. They reported that, the frequency of callus induction increased with increasing concentration of auxins, the optimal levels being 3.0 mg/l of IAA or NAA and 0.5mg/l of Kin. The immature leaf was found to be most efficient explant in producing callus.

Yuan *et al.* (1997) cultured shoot tip and hypocotyl segments of 7 to 10-days-old seedlings of flax cultivars 7309, Shuangya 1 and Shuangya 5 on N_6 and B_5 media supplemented with 4 mg/l IAA, 2 mg/l kinetin and 200 mg/l LH. They reported that explants of cv. 7309 had the highest callus induction rate 92.3% and explants of Shuangya 5 had the lowest rate 29.5%.

Dedicova *et al.* (2000) cultured hypocotyl and cotyledon explants of fibre flax cv. Carolin and Alex and linseed cv. Szegedi-30 and Flanders on MS or MO medium supplemented with 0 or 1 mg/l

NAA + 0, 0.5 or 1 mg/l BA or with 0.25, 0.5 or 1 mg/l TDZ, for callus production. Hypocotyl explants were pretreated with 2, 5 or 10 mg/l 2, 4-D for 24 or 72 h and cultured on MO medium supplemented with 2 mg/l of kinetin, BA or zeatin or 1 mg/l each of BA and NAA. They reported that cotyledon explants produced higher callus as compared with hypocotyl explants.

Tejavathi *et al.* (2000) reported that calli of *Linum usitatissimum* cultivars R552, Jeenan and Gaurav were initiated from hypocotyl explants which cultured on MS medium with auxin 2,4-D (2.26-22.62 μM), IAA (2.85-28.54 μM) or NAA (2.69-26.85 μM) or a combination of auxin and BA (2.22 μM). Profuse friable callus was observed on explants cultured on MS + 2, 4-D (2.26-18.1 μM) or IAA (2.85-28.54 μM). But no differentiation of embryos was observed. Cultures of linum cv. Jeevan and Gaurav required lower concentrations of NAA (2.69-10.74 μM), while R552 needed higher concentrations of NAA (10.74-21.48 μ M) for callus production.

Shiv-Ratan *et al.* (2001) investigated the effect of cotyledon and hypocotyl explants excised from 9-days-old seedlings of flax cultivars RC 781, T 59, Pusa Bold, PHR 1 and RH 78 on callus production. The explants were cultured onto MS medium supplemented with growth regulators IAA (0.2 mg/l), BA (2.0 mg/l), 2,4 -D (4.0mg/l), IAA + BA (0.2 mg/l each), IAA + kinetin (0.2mg/l each) or 2,4-D + BA (4.0 + 2.0 mg/l). They reported that MS medium supplemented with IAA + kinetin, gave the best result of callus production as compared with other supplementation.

Blinstrubiene *et al.* (2004) studied the effect of two different types of explants: stem segments and hypocotyls of three genotypes of flax on callus production. The explants were cultured on nutrient MS media differing in their macro and micro salt concentrations and different levels of growth regulators. They reported that callus induction was possible with all used types of nutrient media. They also studied some factors affecting on *in vitro* callogenesis capacity of oilseed flax cultivars Lirina, Barbara and Szaphir. Genotype differences were demonstrated on three media which differed in levels of growth regulators. They reported that MS medium supplemented with 1.0 mg/l BA +0.1 mg/l NAA was more suitable for *in vitro* callogenesis. Moreover, they reported that callogenesis was influenced not only by exogenous hormones but also by the type of explant. Leaf of all tested genotypes was more responsible for callogenesis than stem explants on all cultured media. Callus was derived at the same time from stem explants in either tested culture media with 1.0 mg/l BA + 0.1 mg/l NAAl or 5.0 mg/l BA +0.5 mg/l NAA.

Yurong *et al.* (2004) investigated the effect of culturing of flax anthers at 35 °C for one to four days prior to culturing at 25 °C in darkness, genotype and anther orientation on percentage of callus induction. They reported that culturing of anthers at 35 °C prior to continuous culture at 25 °C in darkness did not significantly improve the percentage of anthers producing calli. However, the genotypic effects were significant on the percentage of anthers producing calli.

Bohu *et al.* (2005) screened wide range of flax *Linum usitatissimum* L. cultivars for *in vitro* callus production from anthers.