Czech University of Life Sciences, Prague

Faculty of Agrobiology, Food and Natural Resources

Department of Horticulture

In vitro selection for *Fusarium oxysporum f.* Sp. *conglutinans* resistance in *Brassica* vegetables

Diploma Thesis

Author: J. A. M. Oushadee A. J. Abeyawardana. BSc.

Supervisor: Ing. Martin Kaudela. Ph.D

2017

DECLARATION

I declare that the Diploma Thesis, *In vitro* selection for *Fusarium oxysporum f.* Sp. *conglutinans* resistance in *Brassica* vegetables, is my own work and all the sources I cited in it are listed in Bibilography.

Prague, 2017 Signature:

ACKNOWLEDGEMENTS

First, I would like to express my immense honorable gratitude to my supervisor Ing. Martin kaudela, Ph.D, Head of the Department of Horticulture, Czech University of Life Sciences in Prague. I am deeply thankful to him for his valuable support and guidance throughout my research study. I am also appreciated and indebted for encouragement given to me during my study.

Secondly, I would like to thank Mgr. Marie Kafková, Study coordinator for MSc studies, Facultu of Agrobiology, food and Natural Resources, Czech University of Life Sciences in Prague for guidance and support given me during whole study programme. I also thankful to Ing.Véra Kofránková and Ing. Tomáš Jekínek who are Ph.D students of the Department of Horticulture for the support given during my research study.

I would like to thank all my friends Adéla, Cristina, Rathane and Pavel for all support given to me since beginning of the research study.

Finally, I am thankful and gratitude to my parents for encouragement and advises given throughout my study to see valuable output.

SUMMARY

Fusarium wilt is a concerning problem in *Brassica* vegetable cultivation. As economically and nutritionally important, widely growing *Brassica* vegetable worldwide, it is important to control the damage cause by *Fusarium* wilt to the production. As most reliable control is cultivation of *Fusarium* resistance cultivars, development of new cultivars carrying Fusarium resistance is timely important effort. In this study, development of resistance to *Fusarium oxysporum f.* Sp. *conglutinans* in six cultivars of *Brassica oleracea* var. *capitata* plants by *in vitro* chemical mutagenesis and selection, through direct and indirect organogenesis was studied.

Regeneration capacity of explants (six days and ten days old hypocotyl, shoot tip and cotyledon) excised from *in vitro* germinated seedlings, through direct and indirect organogenesis path ways were tested before undergoing chemical mutagenesis for selecting explants having better responses in *in vitro* environment. Six days and ten days old hypocotyl, shoot tip and calli derived from 6 days old hypocotyl explants on the MS medium supplemented with NAA (0.2 mg/L), BAP (3 mg/L) and $AgNO_3$ (0.5 mg/L), were subjected to chemical mutagenesis treatment (DMSO (4% (v/v)) and EMS (0.3% (v/v)), 2 hours at 28 ± 2°C). Shoots developed directly from hypocotyl and shoot tip explants (in the MS medium supplemented with NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), $AgNO_3$ (0.5 mg/L) for shoot induction and NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L) for development) after mutagenesis treatment and calli following mutagenesis treatment were screened for *Fusarium* resistance using *Fusarium* culture filtrate with 15% and 20% v/v selection strength for 30 and 60 days of selection periods in each strength. All six cultivars tested, developed plantlets which showed resistance to *Fusarium* culture filtrate in *in vitro* conditions with different survival frequencies ranging between 12.5% - 84.0 % from hypocotyl and 0.0% - 86.7% from shoot tip explants among cultivars indicating development of resistance to *Fusarium* by *in vitro* chemical mutagenesis. Furthermore, study results were indicated indirect organogenesis and 10 days old hypocotyl and 6 days old shoot tips are potential explants for *in vitro* chemical mutagenesis of *B. oleracea* var. *capitate.*

Key words: *Fusarium*, *Brassica*, cultivar, *in vitro* mutagenesis and selection, resistance

INDEX

ACKNOWLEDGMENT iii

SUMMARY iv

CHAPTER 01: INTRODUCTION

1.1.1 Introduction ……………………………………………………………………...2

1.1.2 Objective and Hypothesis ………………………………………….……….3

1.2 Literature overview ……………………………………………………………..4

1.2.1 Cabbage (*Brassica oleracea* L. var. *capitata*) …………………………...4

1.2.2 *Fusarium oxysporium f.* sp. *Conglutinans* ………………………………5

1.2.2.1 Biological effect of *Fusarium* in the crop ……………………...6

1.2.3 Natural resistance in *Brassica* against *Fusarium* ………………………...7

1.2.4 Conventional mutation breeding …………………………………………...8

1.2.5 Plant tissue culture on mutation breeding ………………………………10

1.2.6 *Brassica* plant tissue culture ……………………………………………11

1.2.7 Genetic variability in the *in vitro* system ………………………………12

1.2.8 *In vitro* mutation induction ……………………………………………..14

1.2.8.1. Physical mutagens …………………………………………...15

1.2.8.2. Chemical mutagens …………………………………………17

1.2.9 *In vitro* mutation selection ……………………………………………...18

1.2.9.1 Selection agents in disease resistance screening ……………...19

1.2.9.2 Culture Filtrate as selection agent……………………………20

1.2.10 *In vitro* mutagenesis in *Brassica* ……………………………22

CHAPTER 02: MATERIALS AND METHOD

2.1 Materials ……………………………………………………………………24

2.1.1 Biological materials ……………………………………………………24

2.1.1.1 *Brassica* seeds ………………………………………………..24

2.1.1.2 *Fusarium oxysporium f.* sp. *Conglutinans strain* ……………..24

2.1.2 Chemicals …………………………………………………………...24

2.1.2.1 For media preparation ………………………………………..24

2.1.2.2 For seed surface sterilization ……………………………...24

2.1.2.3 For mutagenesis treatment …………………………………...24

2.1.3 Growth regulators ……………………………………………………...25

2.1.4 Equipments used for the media preparation, tissue culture and mutagenesis processes …………………………………………………………...25

2.2 Methodology ……………………………………………………………..26

2.2.1 Summary of the study, developing *Fusarium* Resistant *Brassica oleracea L. var. capitata L.* plants through *in vitro* chemical mutation induction..26

2.2.2 Development of cabbage plantlets through *in vitro* plant tissue culture ...27

2.2.2.1 Summary…………………………………………………...27

2.2.2.2 Media preparation and glass ware sterilization ……………….28

2.2.2.3 Direct and indirect shoot regeneration ………………………..28

2.2.2.3.1 Surface sterilization of seeds ……………………….28

2.2.2.3.2 *In vitro* seed germination ...28

2.2.2.3.3 Direct shoot induction ...28

2.2.2.3.4 Indirect shoot induction 29

2.2.2.3.4.1 Callus induction 29

2.2.2.3.4.2 Shoot induction from calli ...29

2.2.2.3.5 Shoot development 29

2.2.2.3.6 Regeneration of roots 30

2.2.3Development of *Fusarium* resistance cabbage plants ...30

2.2.3.1 Preparation of *Fusarium* fungus culture filtrate 30

2.2.3.2 Determination of optimum pathogenic concentration of culture filtrate for selection ...31

2.2.3.3 Application of chemical mutagenesis treatment ...31

2.2.3.4 Selection of calli and shoots carrying *Fusarium* resistance mutations 31

2.2.4 Acclimatization of in vitro derived plants 32

CHAPTER 03: RESULTS

3.1 *In vitro* seed germination and explant excision ...34

3.2 Determination of regeneration efficiency ...35

3.2.1 Direct shoot induction and development 35

3.2.2 Indirect shoot induction and development....40

3.2.3 Root induction 44

3.3 Development of *Fusarium* resistance cabbage plants ……………………………46

3.3.1 Determination of optimum pathogenic concentration of fungus culture filtrate …………………………………………………………..46

3.3.2 Development of resistant plants through direct organogenesis …………49

3.3.2.1 Chemical mutagenesis and *in vitro* selection……………………49

3.3.3 Development of resistant plants through indirect organogenesis ……….55

3.3.3.1Chemical mutagenesis and *in vitro* selection ……………………55

3.3.4 Root induction and acclimatization of *in vitro* developed resistant cabbage plants ………………………………………………………………….61

CHAPTER 04: DISCUSSION AND CONCLUSION

4.1 Discussion …………………………………………………………..64

4.2 Conclusion ……………………………………………………………72

BIBLIOGRAPHY……………………..……………..…………………........................73

LIST OF ABRIVIATIONS ……………………………………………………...87

APPENDICXES ………………………………………………………………….89

LIST OF FIGURES ……………………………………………………………...93

LIST OF TABLES ……………………………………………………………….94

CHAPTER 01

Introduction

CHAPTER 01

INTRODUCTION

1.1.1 Introduction

Brassica oleracea L. var. *capitata* (cabbage) is an economically important vegetable crop grown all over the world and is one of the major species belongs to family *Brassicacea*. Cabbage is an excellent source of crude fibers, vitamins (vit. C, K, A), minerals (Ca) and Folic acid (Telekar and Griggs, 1981; Bjorkman et al., 2011). It also has some other important properties such, free radicals scavenging activity by production of anti- oxidant phenolic compounds, ferric – reducing ability and inhibition of lipid peroxidation (Llorach et al., 2003). Also, Kirsh et al., 2007 and Bjorkman et al., 2011 have reported that cabbage contains many other nutrients which help to prevent wide range of diseases including some cancers and heart diseases.

Fusarium wilt is a major and soil born disease caused by fungus *Fusarium oxysporum* sp. *conglutinance* (FOC) resulting crop damage leading to plant death affecting to the production in cabbage cultivation especially in disease susceptible varieties resulting sever losses of yield and the quality. Discoloration of vascular tissues of the plant is a characteristic feature of this infectious disease and yellowing of lower leaves with gradual spread towards the tip is general symptom of this diseases. Often this occurs on one side of the plant and with time entire plant can be yellow, wild and die. Fungus is mainly infected through wounded roots. Therefore, seedlings are become easy candidate for infection after transplanting and surviving plants become stunted due to root decay.

Cultivation of resistant varieties has better control to the diseases while chemical and other traditional controlling methods are being ineffective in disease control (Arden, 1979; Keinath et al., 1998; Farnham et al., 2001) after fungus has become established in the field. Plant breeding programs for developing resistant varieties against *Fusarium* was started in the United States at the beginning of the 20th century (Walker et al., 1927; Anderson, 1933). Some cabbage cultivars have naturally carrying resistance against *Fusarium* infection and disease development. In conventional plant breeding programs these natural inherited resistant traits are used to develop new varieties with higher level of resistance capacity and it is a time-consuming requiring many years to achieve desired level of resistance following back crossing.

The plant breeding is affected by the limited availability of desired suitable genes in the germplasm basis and most of genetic variations used are naturally occurred. It is important to expand the genetic variability by means of modern technologies. In this case, *in vitro* tissue culture techniques and induced mutagenesis provide great platform and opportunity to broaden the genetic variations in crop improvement. Van den Bulk (1991) has pointed out that the importance of *in vitro* tissue culture application in disease resistance breeding. *In vitro* mutation induction and selection may speed up the breeding program enhancing the rapid recovery of desired variation which couldn't find in nature before in the gene pool or has been lost during the evolution (Brunner, 1995). *In vitro* mutation induction and selection are becoming popular in developing disease resistance crop varieties and acquired resistance through *in vitro* techniques would be horizontal resistance characteristics and would be difficult to be broken by the emergence of new races and be maintaining for a longer period. Therefore in vitro mutagenesis and selection provide efficient, fast and reliable platform for development of new traits in resistance breeding programs of cabbage.

1.1.2 Objective and Hypothesis

The objective of present study is development of *Fusarium oxysporum* sp. *conglutinance* resistant in *Brassica oleracea* L. var. *capitata* (cabbage) using *in vitro* culture technique through *in vitro* chemical mutagenesis using the effect of the mixture, Dimethylsulphoxide (DMSO) and Ethyl methanesulfonate (EMS) and selection, by means of fungus culture filtrate. Efficiency of resistance development was evaluated in six cultivars of *Brassica oleracea* L. var. *capitata,* three different types of explants and two types of *in vitro* regeneration methods (direct organogenesis and indirect organogenesis).

According to my knowledge there are not published studies done for *in vitro* chemical mutagenesis and *Fusarrium* resistance development in six cultivars of *Brassica oleracea* L. var. *capitata* (cabbage) which were selected for this study.

Hypothesis: Resulting new genotypes of *Brassica oleracea* L. var. *capitata* through *in vitro* mutation induction and selection are more resistance to *Fusarium oxysporum* sp. *conglutinance.*

1.2 Literature overview

1.2.1 Cabbage (*Brassica oleracea* L. var. *capitata*)

Cabbage (Brassica oleracea L. var. capitata) is a most important vegetable crop grown globally, belongs to family Brassicaceae. Cabbage was presumably originated in Western Europe from wild types growing on the Mediterranean and Atlantic coasts of Europe (Leike H., 1988) and is the first domesticated Brassica crop. Figure 1.1 shows main growing regions and regions of originated. There are diverse types of cabbage cultivars varying with head size, shape, color and texture.

Figure 1.1: Cabbage cultivating regions and regions of origination in the world (Region of origin: ■ Region of cultivation: ■)1

Cabbage is referred as a low calorie but nutritionally valuable crop as a vegetable containing fiber, vitamins (ascorbic acid, a-tocopherol and b-carotene) and minerals (Ca, K) (Telekar and Griggs, 1981; Prior and Cao, 2000; Bjorkman et al., 2011) and as a medicinally valued crop in traditional medicine for treating headaches, gout, diarrhea and peptic ulcers and in treatment of minor cuts and wounds and mastitis (Cheney, 1950) due to having anti-inflammatory and antibacterial properties (Roy et al., 2007; Ayaz et al., 2008). Numerous studies have shown cabbage produce different phytochemicals such as indole-3-carbinole (I3C), sulforaphane and indoles which have antioxidant activities as free radical scarving activity, ferric reducing ability and inhibition of lipid peroxidation and many other chemicals that can eliminate many diseases from cancer to heart diseases

[1] Schuchert W. Head cabbage (Brassica oleracea L. var. capitata) <**https://s10.lite.msu.edu/res/msu/botonl/b_online/schaugarten/Brassicaoleraceavarcapitata/Cabbage.html** > 18//09/2016

(Byers and Perry, 1992; Prior and Cao, 2000; Brooks et al., 2001; Llorach et al., 2003; Krish et al., 2007; Bjorkman et al., 2011).

Cabbage is more favor to grow best under cool and moist weather conditions (Thompson, 2002) and can grow wide range of soil types with adequate fertilizer application. According to latest reports of FAO 2014, there was 71,778,764 tons of production of collectively cabbage and other Brassica vegetables. However, the cultivation, productivity and quality of cabbage are severely affected by different biotic and abiotic stress factors. Biotic stresses are caused by different insects (i.e. cut worm, cabbage butterfly) and by infections of bacterial and fungal pathogens. Fusarium oxysporum f. sp conglutinans is the destructive fungal pathogen caused Fusarium wilt in cabbage which causes severe loses to cabbage yield worldwide.

1.2.2 *Fusarium oxysporium f.* sp. *Conglutinans*

Fusarum oxysporium f. sp. *Conglutinans* is an ascomycete fungus which contains two pathogenic strains as race 1 and 2 causing wilting or yellowing especially of *Brassica* species (Booth 1971; Armstrong and Armstrong 1981; Ramirez – Villupadue et al. 1985) invading through roots and colonizing in xylem tissues (Tjamos and Beckman.1989). The race 1 causes wilt of cabbage and a wide range of *Cruciferase*. Race 2 causes cabbage yellow in cultivars resistant to race 1[2]. These two strains are closely related and morphologically indistinguishable from other *F. oxysporium* species. Microconidia are one or two celled measures 6-15 × 2.5- 4 µm. Mcroconidia are two to four septate, oval or reniform and formed in false heads[3]. Race 1 can be found globally everywhere, but race 2 has been only found in United States and Russia (Ramirez – Villupadue et al. 1985; Morrison et al. 1994).

This is a soil born root infecting fungus and produce chlamidospores during their life cycle that enables to survive for many years in soil (Couteaudier and Alabouvette 1990). Soil temperature is the main crucial factor for the activity of this fungus. Race 1 is active at soil temperature over 18 ^{0}C and reaches to maximum growth rate between 26 ^{0}C to 32 ^{0}C[2,i] while race 2 is active at low temperature at 12 ^{0}C[2].

[2] Taxa description:Fusarium oxysporum Schlecht. f.sp. conglutinans (Wollenw.) Snyder & H.N. Hansen Amer. J. Bot. 27: 66 1940 < http://www.mycobank.org/BioloMICS.aspx?TableKey=14682616000000063&Rec=16805&Fields=All> 14/07/2016

[3] Pathogen of the month; September 2009. Australasian Plant Pathology Society. <http://www.appsnet.org/Publications/potm/pdf/Sep09.pdf> 20/08/2016

This fungus can be spread in numerous ways. It can produce spores both outside and inside the plants. Thus, the principle method of distribution is infected seedlings and by infected soil, clinging to transplants, tractors and farm equipment wheels, plant debris and drainage water. Having ability to survive in the soil for many years without a host, conventional control methods such as crop rotation, seed treatment, fungicide has very low effect as fungus has established in the field (Arden, 1979; Keinath et al., 1998; Farnham et al., 2001). Use of resistant cultivars is the only practical effective control against *Fusarium* effect.

1.2.2.1 Biological effect of Fusarium in the crop

F. oxysporum is referred as a hemibiotrophic pathogen due to having both biotrophic and necrotrophic phases in its infection cycle. Infection process is started as a biotrophic pathogen, invading roots and moves towards the vascular tissues. When F. oxysporum reaches the vascular tissues; the organism spreads through the vascular tissues exclusively by hyphal proliferation and colonizing in the xylem and producing microconidia (Czymmek, 2007). In the last phase of infection process, F. oxysporum turns into necrotrophic pathogen resulting leaf necrosis, lesion development, wilting and eventual death of plant.

There are many studies carried out to identify the biological activity of Fusarium infection and disease development using Arabidopsis thaliana as model plant and many other plants such as banana, tomato, melon. Fusarium secretes many hydrolytic enzymes, such as cutinases, cellulases, pectinases, and proteases and produces phytotoxins and hormone like compounds (Knogge, 1996), including fusaric acid (Bacon, 1996) and abscisic acid during its infection and progress of the pathogenic cycle in the plant. Hydrolytic enzymes help to break down cell walls and plant defensive mechanisms against *Fusarium*. For an example, Fusarium oxysporum secrete a tomatine (which is an antifungal and antimicrobial agent in tomato plants) degrading enzyme; tomatinase (Lairini et al., 1996). According to many previous studies fusaric acid plays crucial role developing symptoms in the plant. Bouizgarne (2006) showed that fusaric acid alters membrane potential and according to Telles- Pupulin (1996) it is altered permeability of membrane and inhibits mitochondrial oxygen uptake and malate oxidation. Dong et al. (2014) showed that senescence of infected banana plants was induced by Fusarium infection with fusaric acid. F. oxysporum also secretes phototoxic compounds cause root cell collapse, chlorosis in the leaves and reprogrammed the host genes to accelerate senescence (Schenk et al. 2005, Dong et al. 2014).

One of the main visible symptoms; wilting is caused by accumulation of fungal hype in the xylem blocking the xylem vessel leading to water stress, toxins produced by *Fusarium,* host defense responses (Beckman 1987). The water stress caused by xylem blocking leads to leaf epinasty, wilting, chlorosis, necrosis, abscission and severe infection results plant death while lesser infected plants become stunted and unproductive (MacHardy and Beckman, 1981). Vascular browning is the most prominent internal symptom can be seen in Fusarium infection (MacHardy & Beckman 1981). F. oxysporum produces bioactive jasmonates (JA) which promotes senescence in the plants (Cole et al., 2014) and effect on the host JA signaling pathway promoting the establishment of the disease (Thatcher et al., 2009).

1.2.3 Natural resistance in *Brassica* against *Fusarium*

Naturally plants show defense mechanisms themselves against pathogens showing resistance or tolerance the disease. These mechanisms are related with many genes expressions which are defense-related, pathogenesis-related genes, resistance (R) genes; activating different signaling pathways; such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathway which trigger the disease resistance. Previous studies have been shown, the degree of vascular colonization of *Fusarium* and the resistance capacity are quantitatively correlated to the resistance for vascular colonization of the fungus (Smith and Walker, 1930; Anderson and Walker, 1935; Gao et al., 1995 a,b).

In cabbage, two types of resistance can be seen against *Fusarium* wilt; type A and type B (Walker, 1930; Walker and Blank, 1934). Type A resistance is very stable and effective against race 1 inheriting through single dominant resistance gene (Walker, 1930; Walker and Hooker, 1945; Farnham et al., 2001; Pu et al., 2012; Lv et al., 2011, 2014) and this can be used in conventional resistance breeding processes. Type B resistance inherent as polygenic type and is unstable influencing by high temperature breaking the resistance. These type A and B resistance are not effective for *Fusarium oxysporum* sp. *conglutinance* race 2 and the reason for this susceptibility is unclear still.

Even though complete biochemical mechanism of *Fusarium* resistance in *Brassica* is not fully understood, Xing et al. (2016) has observed SA and JA dependent *Fusarium oxysporum* sp. *conglutinance* (FOC) resistance mechanisms in *Brassica oleracea* with related to the expression of number of genes. They identified the candidate FOC resistant gene; comp30068 c1 belongs to TIR-NB-LRR- type resistant gene in roots which is activated the SA- dependent

signaling pathway inducing some other genes encoding pathogenesis- related (PR) proteins in cabbage resistance responses against FOC. During SA dependent pathway, it triggers the expression of numerous genes belongs to two groups; Group 1, that express early in the hypersensitive resistance responses (HR) such as glycosyltransferase and glutathione S-transferase synthesis and Group 2, that express lately in systemic acquired resistant (SAR) such as PRs. Xing et al., (2016) reported that, SA-depended SAR is involved in defense responses in cabbage against *Fusarium oxysporum* and the activation of this SA-depended defense pathway is occurred against the biotrophic phase of the *F. oxysporum* (Glazebrook, 2001).

Another study, Lv et al. (2014) and Shimizu et al. (2015) have reported re-Bol037156, putative TRI- NBS-LRR type gene as the candidate gene for *fusarium* wilt resistance in cabbage. Many studies have suggested as JA dependent signaling pathway is important for *Fusarium* resistance. According to Dong (1996), JA-mediated disease resistance path way is distinct from the SA-mediated SAR. Moreover, *Brassicaceae*-specific glucosinolates-myrosinase system also plays a key role in Fusarium resistance in Brassicaceae. Xing et al., (2016) demonstrated the activation of Myrosinase gene (comp35843_c0) and JA-induced myrosinase-binding proteins (MBPs) gene (comp41986_c0) during fungal infection and it is JA-mediated signaling pathway is contributed for this process leading to FOC resistance in cabbage. Myrosinases (Brassicaceae-specific β-glucosidases) hydrolyze glucosinolates (which are defense compounds in Brassica crops) (Asai et al., 2002; Nagano et al., 2008; Rask et al., 2000) to aglucons and it is decomposed into toxic compounds such as isothiocyanates, thiocyanates, nitriles and epithionitrile which are important for disease resistance (Mithem et al., 1992). Myrosinases make complexes with myrosinase-binding proteins (MBPs) and responsible for hydrolysis of glucosnolates. Xing et al., (2016) suggested that these signaling pathways are not independent and comprise a complex resistance network in response to FOC infection. Shimizu et al., (2015) and Lv et al., (2014) showed that there are insertion/deletion variations of the candidate FOC resistance gene between resistant and susceptible cabbage and resistance gene has constitutive expression in resistance materials.

1.2.4 Conventional mutation breeding

Since people were interested on crop improvement, controlled and selective cross breeding and selection of variable plants with desirable best performance is carried out in the field. This process is highly time consuming and demanding to obtain a new crop variety which is ready

cultivate in the field. Because, cross breeding is based on the sexual reproduction and several steps of backcrosses are required to achieve desired unique combination of the DNA/ traits (for examples, disease resistance, high yield, drought/salt resistance etc) eliminating undesirable traits from parent plants. As Kuckuck *et al.* (1991) and Villalobos & Engelmann (1995) have been showed that the availability of genetic diversity including desired characters is important in successful breeding programme. But the success of this breeding is largely limited into same species due to far hybridization barriers and number of available traits within the species.

Mutation breeding with induced mutagenesis has come in to account with many comparative advantages advancing the plant breeding programme. This is quick, transferable and ubiquitously applicable. According to joint FAO/IAEA mutant varieties database and Ahloowalia et al. (2004), there are more than three thousand plant varieties belong to more than two hundred plant species (Figure 1.2) developed by mutation breeding have been officially released from more than seventy countries to use either as parents for new variety development or for direct cultivation.

There are three types of mutagenesis which are employed in mutation breeding; induced mutagenesis (by utilization of physical or chemical mutagens), site-directed mutagenesis and insertion mutagenesis (Oladosu et al., 2016). In the conventional mutation breeding, mutations are induced by subjecting seeds, seedlings or vegetative propagules (such as bulbs, tubers, corns, rhizomes) (Wani et al., 2014) to mutagenic agents.

Concerning the breeding of *Brassica* crops, hybridization with wild *Brassica* species is frequently used to improve disease resistance and environmental tolerance (Sretenović-Rajičić, 2004). Currently, breeders attempt to use mutation breeding to improve *Brassica* crops (Vinterhalter et al., 2007). There are many reported studies carried out for induced mutagenesis as tool for developing variations in improved *Brassica* breeding.

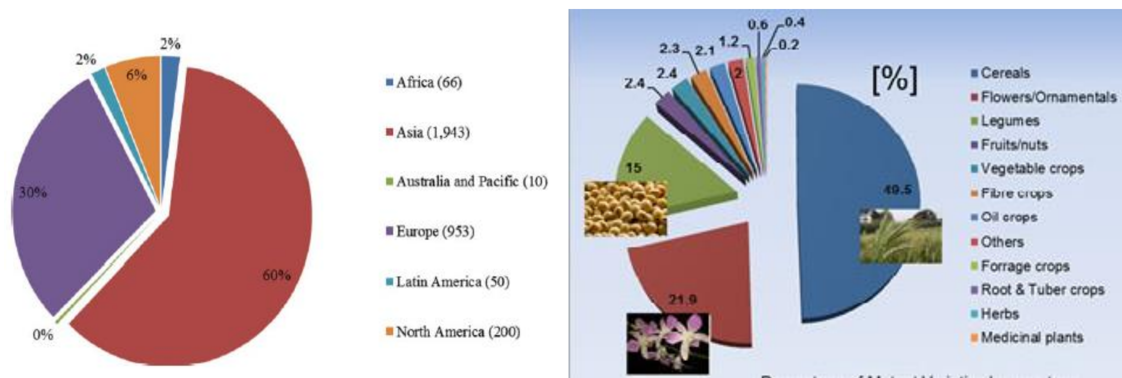


Figure 1.2: Distribution of mutant crop varieties by continents (Oladosu et al., 2016) and percentage of mutant varieties have been officially released. (FAO/IAEA)[4]

Mizuno et al. (2014) has isolated 293 independent M_2 mutant lines of *Brassica rapa* by exposing seeds to EMS (ethyl methanesulfornate). They observed three main phenotypic mutations; early flowering, crane-like and rosette among those isolated mutant lines. Das and Rahman (1988), Shah *et* al. (1990), and Das *et al.* (1999) have isolated mutants of *Brassica rapa* and *Brassica napus* with high yield potential after induced mutagenesis and Javed *et al.* (2003) also has selected high yielding mutants of *B.campestris* var. *toria* through gamma rays irradiation (reviewed in Sindh et al., 2015). Zareen and Pratibha (1995) has reported that mutagenic effectiveness and efficiency of using gamma rays, diethyl sulphonate and maleic hydazide in mutagenesis of *B.campestris.* Schnurbusch *et al.,* (2000) and Haque (2002) studied oil seed mutants with increased palmitic acid and mutant of *B. juncea* with reduced linolenic acid respectively (reviewed in Sindh et al., 2015). Tomlecova et al., (2006) studied chemical mutagenesis and DNA polymorphism in Brassica oleracea var. capitata L. cv. Ditmarsko.

1.2.5 Plant tissue culture on mutation breeding

Nowadays, plant tissue culture along with the biotechnology plays an important role in plant breeding with plant quality enhancements. Tissue culture increases the efficiency of achieving the desired objective which is not possible to achieve through the conventional breeding as by far cross hybridization. Furthermore, it is shortened the breeding cycle and overcome problems arise in conventional breeding due to seed dormancy. Protoplast culture, somatic hybridization, embryo/ovary/ ovule culture, anther/microspore culture, *in vitro* pollination, somatic

[4] Mutation breeding. FAO/LAEA programme. < http://www-naweb.iaea.org/nafa/pbg/mutation-breeding.html> 12/12/2016

embryogenesis, *in vitro* mutagenesis and genetic transformations are important in *in vitro* tissue culture techniques utilize in plant breeding with enhanced characters. *In vitro* mutagenesis and somaclonal variations in *vitro* cultures, help for better and easy achievements in *in vitro* production of mutant lines in point of breeding generating novel genetic variability (Larkin and Scowcraft, 1983; Brar and Jain, 1998). Because of relatively high mutagenic rate and useful mutants developed from the stable genetic background, *in vitro* mutagenesis has become more advanced very fast in mutational breeding (Ma & Zhao, 1998; Castillo et al., 2001; Barro et al., 2001, 2003).

Somaclonal variations which arise in *in vitro* cultures itself during reprogramming of plant development from part of plant tissue, also produce novel genotypes which have interest in plant development (Gopi and Ponmurugan, 2006). Artificially mutations can be induced in the genome by application of mutagenic treatments and mutant plant with desired characters can be selected either by genetic markers, metabolic or biochemical differences etc.

Another one of advantages of plant tissue culture on mutational breeding is independence on seasonal changes allowing year-round propagation and screening and facilitating more stable characters such as biotic and abiotic resistances, increased production of important biochemical plant products. This also facilitate the manipulation of large population of mutagenic plants to be selected and clone selected plants.

Even though, this technique has numerous powerful advantages on mutational breeding, the main disadvantage of this is requirement of optimum regeneration protocols on a species-by-species manner that can be successfully applied for the breeding program.

1.2.6 *Brassica* plant tissue culture

Plant tissue culture has become as an important tool in genetic engineering and crop improvements. Having an efficient *in vitro* tissue culture protocol is a prerequisite for plant improvement through genetic engineering or *in vitro* techniques. Different culture protocols have been optimized for different plant species successfully. Generally, *Brassica* is referred as recalcitrant to *in vitro* plant tissue culture (Zhanget al., 1998). However, there are many reports on the successful application of *in vitro* culture techniques (i.e. organogenesis and somatic embryogenesis) for regeneration of different *Brassica* spp (Antonio et al., 1987; Jain et al., 1988; Ono et al., 1994; Koh and Loh, 2000; Khan et al., 2002; Sparrow et al., 2004; Munshi et al., 2007; Pavlović et al., 2010; Yu et al., 2010; Mollika et al., 2011) and for improvement of

Brassica crops through genetic transformation ((Metz et al., 1995; Bhalla and Smith 1998; Jin et al., 2000; Li et al., 2005; Sretenović-Rajičić et al., 2006 and 2007; Bhala and Singh, 2008).

In vitro plant regeneration is depended on several factors including plant genotype, type of explant, media composition, hormones, environmental conditions (temperature, light, humidity) etc ((Bhalla and Smith, 1998; Zhang et al., 2005; Liu et al., 2010; Maheshwari et al., 2011). Plant regeneration of different varieties of *Brassica oleracea* through different explants has been reported. For examples; leaf and root segments (Cao and Earle, 2003), hypocotyls (Puddephat et al., 2001; Hazrat et al., 2007; Qamar et al., 2014), cotyledons (Dale and Ball, 1991; Hazrat et al, 2007), peduncle explants (Christey and Earle, 1991), protoplast culture (Delpierre and Boccon-Gibod, 1992; Yang et al., 1994), curd and stem (Bhalla an Weerd, 1999), shoot tip (Handayani, 2014) and anther culture (Yang et al., 1992). However, some variation and various levels of regeneration efficiencies were observed by these study groups even for same varieties.

With respect to *B. olereacea* var. *capitata*, successful *in vitro* plant regeneration has reported hypocotyls, cotyledons, roots, leaves, peduncle segments, thin cell layers, protoplasts and immature zygotic embryos (reviewed in Cardoza and Stewart, 2004; Munshi et al., 2007; Kiełkowska and Adamus, 2012; Pavlović et al., 2013; Ravanfar et al., 2014; Gerszbery et al., 2015).

According to previous studies, *Brassica* plants carry a set of genes (CC) which are responsible for shoot formation ((Murata and Orton, 1987; Narasimhulu and Chopra, 1988; Ono and Takahata, 2000; Sparrow et al., 2004b) and *in vitro* shoot regeneration is strongly controlled by those genes. Furthermore, the *in vitro* regeneration of *Brassica* is highly genotype dependent and genotype specificity act as a limiting factor of *in vitro* regeneration (Ono et al., 1994; Poulsen, 1996; Cardoza and Stewart, 2004).

1.2.7 Genetic variability in the *in vitro* system

Tissue culture is an efficient technique used in vegetative clonal propagation (micropropergation) and in the commercial basis, having a uniform population is the basic aim of *in vitro* micropropergation. However, within the resulting *in vitro* regenerated population

often contains individuals with variations than others due to the occurrence of uncontrolled and random spontaneous variation during *in vitro* plant regeneration and growth (Karp, 1994). These variations are referred as somaclonal variations and this is a common phenomenon in the plant tissue culture (Skirvin et al., 1993).

Many studies have been carried out to understand the occurrence of somaclonal variations in the *in vitro* cultures. According to Sato et al., (2011b), these variations are mainly resulted by newly generated mutations from tissue culture processes. Early studies have been shown that various stress factors which are associated with tissue culture such as, wounding, exposure to sterilization agents, reprograming of fully differentiated or undifferentiated tissues (protoplasts, calli) (Sahijram et al., 2003; Duncan, 1997), different media compositions (i.e. high concentration of plant growth regulators (auxin and cytokinins), nutrients, sugar from the nutrient medium as a replacement of photosynthesis in the leaves, lighting conditions, the disturbed relationship between high humidity and transpiration (Joyce et al. 2003; Sato et al. 2011b; Smulders and de Klerk, 2011). Furthermore, some other studies have shown that oxidative stress which is resulted by culture initiation and subsequent subculture cycles (Krishna et al., 2008), causes occurrence of variations upon the *in vitro* cultured plants (Cassells and Curry, 2001; Tanurdzic et al., 2008; Nivas and DSouza, 2014). The levels of pro-oxidants or reactive oxygen species (ROS) (superoxide, hydrogen peroxide, hydroxyl, peroxyl, alkoxyl radicals etc) are increased as a result of oxidative stress. These ROS causes alteration of hyper/ hypo- methylation of DNA molecule (Wacksman, 1997), change of ploidy level (from polyploidy to aneuploidy), chromosome breakage, chromosome rearrangements, deletions and substitutions of DNA base (Czene and Harms-Ringdahl, 1995) leading to mutations in the *in vitro* cultured plant tissues. Some growth regulators, such as 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), BAP (6-benzylaminopurine) and synthetic phenylurea derivatives (4-CPPU, PBU and 2,3-MDPU) are considered as responsible for genetic variations in *in vitro* plant tissues (Siragusa et al., 2007; Biswas et al., 2009; Sun et al., 2013; Sales and Butardo, 2014) and extended cultivation duration in mediums containing 2,4-D have an influence on arising higher ploidy levels in callus cells (da Silva and Carvalho, 2014).

The frequency and the nature of somaclonal variations are depended on the tissue culture technique. Zayova et al. (2010), Saravanan et al. (2011) and Duncan (1997) have indicated that callus cultures have higher mutation rate than explants with preexisting meristems (such as axillary buds and shoot tips). The longer culture periods also increase the frequency of

somaclonal variations (Kuznetsova et al., 2006; Gao et al., 2010; Farahani et al., 2011; Jevremovic´ et al., 2012; Sun et al., 2013). In the review of Krishna et al., (2016) has indicated that several studies have been shown, plant genotype is also an important determinant of somaclonal variations (Shen et al., 2007; Tican et al., 2008; Nwauzoma and Jaja, 2013).

These resulting variations can be temporary (epigenetic or physiological effects) or permanent (genetic) changes. Temporary variations are unstable and nonheritable. These can be disappearing either when plants are removed from the *in vitro* conditions or during sexual reproduction (Kaeppler et al., 2000). Permanent and heritable somaclonal variations are used as valuable tool for the genetic improvement of crops. This phenomenon has used developing crops with disease resistant, high quality, high yielding crops etc (Eva et al., 2012). According to Cassells et al. (1998) somaclonal variations have a similar spectrum of genetic variation to induced mutation as both result in qualitative changes.

1.2.8 *In vitro* mutation induction

Having germplasm with desired variations in their genome is necessary in plant breeding. If existing varieties are not carrying such qualities, it is necessary to induce such variations. Naturally in nature, spontaneous mutations are occurred but in very low frequencies. Artificial mutation induction is come forward in this point leading rapid and increased variability in the crop resulting superior plant varieties (Ahloowalia, 1998; Jain, 2005; Maluszynski et al., 1995).

Plant tissue culture and *in vitro* mutation induction techniques have become popular nowadays as an effective tool in mutation breeding with crop improvements (Ahloowalia et al., 2004). Use of plant tissue culture in mutation induction has various advantages in several aspects improving the effectiveness of mutagenesis compared to conventional mutagenesis which has several limitations with availability of a large mutagenized plant population for screening and selection, complex nature of apical meristems and propagating materials in vegetatively propagated plants (Micke et al., 1990). In *in vitro* mutation induction, there is a wide range of plant materials (single cells, buds, tissues, pollen, embryos etc.) that can be selected for the mutagenesis treatment and use of these small plant materials s reduced the risk of chimeric development and has higher probability of mutation expression (D’Amato, 1977). This also facilitates application of uniform mutagenic treatment resulting high mutation frequency and enabled to maintain large population of plant materials in limited space within short time period

and plant materials are kept disease free (Penna et al. 2012). Selection of mutated plants with desired variations and cloning of selected variants are rapid through subculture propagation cycles in *in vitro* process (Ahloowalia, 1998).

Van Harten (1998) has defined mutations as heritable changes in the DNA sequences which are not derived from genetic segregation or recombination. The artificial mutation induction can be done by specific treatments with either physical or chemical mutagens. These mutagens can cause three types of mutations; intragenic (point mutations) which occur within a gene, intergenic (structural mutations) which occur within a chromosome and lastly mutations leading to change of ploidy level (polyploidy, aneuploidy and haploidy) (Pathirana, 2011). Structural mutations can be results of inversions, translocations, duplications and deletion of chromosome. Point mutations or single nucleotide polymorphisms (SNPs) can happen by miss-pairing of the nucleotide bases in the treated DNA during replication, leading transitions either between purine bases or between pyrimidine bases and transversions of purines and pyrimidines (Kharkwal, 2012). These point mutations may result phenotypically visible mutations while most induced mutations are recessive. Frame-shift mutations are also important type of mutations can be happened in the genes DNA through addition or deletion of a nucleotide base pair. This happens when the bases pair with two bases or fails to pair at all with any bases and alter the reading frame of the gene. These frame-shift mutations may completely change the information of the gene starting from the point of deletion or addition (Kharkwal, 2012; reviewed in Oladosu et sl., 2016). In addition, extranuclear (chloroplast and mitochondrial) mutations also can be happened which can have considerable interesting effect to agriculture (Pathirana, 2011).

1.2.8.1. Physical mutagens

The most common physical mutagens used in mutation induction are ionizing radiation; gamma rays (γ-rays), X-rays, Ultraviolet (UV) rays, fast neutrons, alpha and beta particles, proton and ion beams (Table 1.1). Among all radiations used, X-rays and gamma-rays are the most widely using ionizing radiation types. Ionizing radiations carry high energy and can be penetrated deeper into the tissue and cause diverse types of chemical changes (reviewed in Stefano, 2001).

UV radiation has limited use due to low energy and having moderate penetration capacity into tissues comparing to other ionizing radiation (gamma and x-rays) (reviewed in Stefano, 2001).

This moderate penetration capacity can be used for treating leaf tissues (epidermal cells) prior to regeneration induction in order to minimize the risk of arising chimeric mutations (Pinet-Leblay et al., 1992).

Ion beams is a new physical mutagen that can be substituted instead of γ rays and X-rays (Watanabe, 2001; Matsumura et al., 2010). These beams composed of positively charged ions at a high speed and form high linear energy transfer (LET) radiation. This LET radiation can cause chromosomal aberration, lethalty resulting mutations and damages happened to DNA strands are less repairable due to resulting deletion of various sizes of DNA fragments compared to damages caused by ionization radiations (Bughio et al., 2007).

Generally, physical mutagens produce dimmers and reactive ions which cause the damages ranging from aberrations of DNA molecule (breaking the chemical bonds in DNA molecules, deletion/ addition/ substitution of nucleotides) to gross chromosomal breakages and rearrangements (reviewed in Chikelu Mba, 2013). The degree of accuracy and sufficient reproducibility are the major advantages of using physical mutagens with compared to chemical mutagens (Review: Oladosu et al., 2016).

Table 1.1: Common physical mutagens and their characteristics (Review in Oladosu et al., 2016)

Mutagen	Source	Characteristics
X-rays	X-ray machine	Electromagnetic radiation; penetrates tissues from a few millimetres to many centimetres
Gamma rays	Radioisotopes and nuclear reaction	Electromagnetic radiation produced by radioisotopes and nuclear reactors; very penetrating into tissues; sources are ^{60}Co (Cobalt-60) and ^{137}Cs (Caesium-137)
Neutrons	Nuclear reactors or accelerators	There are different types (fast, slow, thermal); produced in nuclear reactors; uncharged particles; penetrate tissues to many centimetres; source is ^{235}U
Beta particles	Radioactive isotopes or accelerators	Produced in particle accelerators or from radioisotopes; are electrons; ionize; shallowly penetrating; sources include ^{32}P and ^{14}C
Alpha particles	Radioisotopes	Derived from radioisotopes; a helium nucleus capable of heavy ionization; very shallowly penetrating
Protons	Nuclear reactors or accelerators	Produced in nuclear reactors and accelerators; derived from hydrogen nucleus; penetrate tissues up to several centimetres
Ion beam	Particle accelerators	Produced positively charged ions are accelerated at a high speed (around 20%–80% of the speed of light) deposit high energy on a target

1.2.8.2. Chemical mutagens

There are different kinds of chemicals used in inducing mutagenesis in plant, which belongs to various mutagenic groups; alkylating agents, base analogues, Nitrous acid, Intercalating agents (acridine orange, proflavin, ethidium bromide), Miscellaneous group of chemicals (e.g., *N*-acetoxy-*N*-2-acetyl-aminofluorine—NAAAF), hydroxylamine etc. The effects these chemical mutagens on DNA molecule is caused through deamination, facilitating transitions and insertions, stopping transcription and replication and breaking DNA strands (reviewed in Chikelu Mba, 2013 and Oladosu et al., 2016). Table 1.2 is showed commonly using chemical mutagens and their action on mutagenesis.

Alkylating agents such as ethylmethane sulfonate (EMS), diethyl sulfonate (DES), ethyleneimine (EI), ethyl nitroso urethane (ENU), ethyl nitroso urea (ENH), methyl nitrosourea (MNH) are the most widely using chemical mutagens. Among those, EMS is referred as very effective mutagen and there are many studies tested its effectiveness in different species. For examples, rice (Bhan and Kaul, 2003), wheat (Bozzini and Mugnozza, 2003), and barley (Nicoloff, 2003), *Arabidopsis thaliana* (Jacobs, 2005), *Saintpaulia* (Fang J, 2011), *Chrysanthemum* (Rodrigo et al., 2004). EMS reacts with nitrogen bases (guanine or thymine) adding an ethyl group which mislead the DNA replication machanism to recognize the methylated bases as an adenine or cytosine.

Table 1.2: Commonly used chemical mutagens (Review in Oladosu et al., 2016)

Mutagen group	Example	Mode of action
Alkylating agents	1-methyl-1-nitrosourea (MNU); 1-ethyl-1-nitrosourea (ENU); methyl methanesulphonate (MMS); ethyl methanesulphonate (EMS); dimethyl sulphate (DMS); diethyl sulphate (DES); 1-methyl-2-nitro-1-nitrosoguanidine (MNNG); 1-ethyl-2-nitro-1-nitrosoguanidine (ENNG); N,N-dimethylnitrous amide (NDMA); N,N-diethylnitrous amide (NDEA)	React with bases and add methyl or ethyl groups and, depending on the affected atom, the alkylated base may then degrade to yield an abasic site, which is mutagenic and recombinogenic, or mispair to result in mutations upon DNA replication.
Azide	Sodium azide	Same as alkylating agents.
Hydroxylamine	Hydroxylamine	Same as alkylating agents.
Hydroxylamine	Hydroxylamine	Same as alkylating agents.
Antibiotics	Actinomycin D; mitomycin C; azaserine; streptonigrin	Chromosomal aberrations also reported to cause cytoplasmic male sterility.
Nitrous acid	Nitrous acid	Acts through deamination, the replacement of cytosine by uracil, which can pair with adenine and thus through subsequent cycles of replication lead to transitions.
Acridines	Acridine orange	Intercalate between DNA bases thereby causing a distortion of the DNA double helix and the DNA polymerase in tum recognizes this stretch as an additional base and inserts an extra base opposite this stretched (intercalated) molecule. This results in frame shifts, i.e. an alteration of the reading frame.
Base analogues	5-bromouracil (5-BU); maleic hydrazide; 5-bromodeoxyuridine; 2-aminopurine (2AP)	Incorporate into DNA in place of the normal bases during DNA replication thereby causing transitions (purine to purine or pyrimidine to pyrimidine); and tautomerization (existing in two forms which interconvert into each other, e.g. guanine can exist in keto or enol forms).

However, this typically does not change the reading frame shifts, but alter the triplet sequence and change of a single base within a coding region can causes either a nonsense codon which stops transcription or an altered codon which changes the amino acid transcribed leading to de-activate, reduce efficiency of the protein or production of a new protein[5].

The efficiency of mutagenesis by chemical mutagens is depended on several factors including chemical and physical properties of the mutagen, solubility of the mutagen, temperature, light, pH of the solution, oxygen availability during the treatment, capacity of absorption, method of application, size of the plant materials, post-treatment washing etc (Nova, 1991). With compared to physical mutagens, chemical mutagens cause more point mutations rather than chromosomal changes and chemical mutagens have greater efficiency than physical mutagens (Rago and Faria, 2001; Jacobs,2005). However, Van Harten, (1998) emphasized the major concern of chemical mutagenesis is low absorption/ penetration into plant tissues leading to reduced mutation efficiency.

1.2.9 *In vitro* mutation selection

The selection of targeted mutants is an integral part in *in vitro* mutagenesis. Plant tissue culture provides a wider range of choice of selections following mutagenesis and *in vitro selection* has higher selection pressure compared to *in vivo* selection (Donovan et al., 1994). However, the efficiency of *in vitro* selection and development of mutant plant are depended on several factors; such as, enormous number of variant cells, way of application of *in vitro* selection method, method of regeneration and inheritability of targeted mutation (Widorentno et al., 2003; Yusnita, 2005).

The ability to select desired mutated trait at cellular level is important in *in vitro* selection process and express those traits in the regenerated plants (reviewed in Penna et al., 2012). Because of this, traits such as height, color, yield etc which are under polygenic control can't be selected at cellular level (Ahloowalia, 1998). In review of Penna et al, (2012) has mentioned

---

[5] *Brown N., Institution of Plant Breeding, Genetics and Genomics, University of Georgia* <http://plantbreeding.coe.uga.edu/index.php?title=20._Mutagenesis> (11/12/2016)

that the traits such; disease resistance, stress tolerance, herbicide tolerance and enhanced nutritional qualities can be selected in *in vitro* techniques.

There are two types of selection techniques that can be applied in *in vitro* selection; single step selection and multi-step selection. In single step selection, selection agent is incorporated into the culture medium at the level of maximum inhibitory concentration (MIC) for several subculture cycles (at least 2-3 subculture cycles). In the multi-step selection, sub-lethal concentration (SLC) that is less than MIC of selection agent is incorporated in to the culture medium and several subculture cycles are maintained with a gradual increase of the selection strength level. Many studies have been suggested that the selected traits through multi-step selection are often more stable and expressive as a result of constant exposing to increasing strength levels of selection agent (Miller and Hughes, 1980; Miao et al., 1983; McCoy, 1987; Patade et al., 2006; review: Penna et al., 2012). Farther more, according to Van den Bulk (1991), the type and the genetic basis of the resistant mutant in selected plants are depended on the selection procedure applied. For an example, selection for disease resistance with the application of host specific toxins may results toxin resistant plants which have complete resistance (qualitative resistance) to the pathogen (Van den Bulk, 1991; Amusa, 2006) while isolating plants carrying incomplete resistance for characters which possibly determined by mutations in minor genes (quantitative resistance) by using increased levels of tolerances (i.e. salt/ drought resistance) (Review in Predieri, 2001).

1.2.9.1 Selection agents in disease resistance screening

Selection of efficient selection agent is important to obtain stable mutant trait (Kumara et al., 2008 a, b). There are many studies reported using different selection agents and the type of selection agents is depended on the desired character need to be selected. Phytotoxins, culture filtrates of pathogenic microbes, high concentrations of metabolites and their analogues, toxic drugs, nutrients or hormones, environmental stresses (salt, drought etc) are some of examples for selection agents that can be used (review in Penna et al., 2012).

In case of selection for disease resistance, there are several applicable selective agents such as; host-selective toxins (HMT-toxin of *Cochliobolus heterostrophus*, PM-toxin of *Phyllosticta maydis*, and AAL-toxins of *Alternaria alternate* ((Buiatti and Ingram, 1991)), nonhost-selective toxins (sirodesmin PL of *Leptosphaeria Maculans* (Šva´bova´ and Lebeda, 2005),

fusicoccin, of *F. amygdali*), living pathogens, chemicals (fusaric acid), pathogen cell wall components (elicitors) (Storti *et al*., 1992; Koike *et al*., 1993b; Koike and Nanbu, 1997). When using, toxins produced by pathogens, it should be considered about that, it should be involved in disease development and the selected toxin must be effective at the cellular level (Hammerschlag, 1984; Yoder, 1980; Šva´bova´ and Lebeda, 2005). Some examples for using different selection agents in *in vitro* selection for disease resistace in crop plants which have done by different study groups are shown in the table 1.3.

There are several studies reported use of live pathogens itself successfully for screening disease resistance *in vitro* i.e. *Xanthomonas campestris* (Hammerschlag 1990), *Clavibacter michiganensis* (Bulk *et al*., 1991), *Alternaria alternata* (Takahashi *et al*., 1992), *Phytophthora cinnamoni* (Cahill *et al*., 1992), *Mycosphaerella musicola* (Trujillo and De Garcia, 1996). However, inoculaton of live pathogens into *in vitro* cultures causes different stress factors leading to death of plant materials (Ingram and Helgeson, 1980; McComb *et al*., 1987; Heath-Pagliuso *et al*., 1988, 1989; Lebeda and Švábová, 1997). Pathogens can overgrow devastating plant materials due to presence of favorable growth conditions (higher humidity, reduced air velocity, media rich in nutrients) in the *in vitro* culture conditions (Lebeda and Švábová, 2010). To overcome this problem, modified selective agents, such as culture filtrates (partially purified toxins, phytotoxic substances, toxic extracts), toxins (pathogen cell-wall components) are used (Švábová and Lebeda, 2005).

1.2.9.2 Culture Filtrate as selection agent

Pathogen culture filtrates have been successful tested in selection of disease resistant plant varieties (i.e. alfalfa: Binarova et al., (1990), chickpea: Singh et al., (2002); Hamid and Strange (2000), carnation: Thakur et al., (2002), cauliflower: Mangal and Sharma, (2002), ickpea (C. arietinum): Rao and Padmaja, (2000), Pea (P. sativum): Švábová and Odstrčilová, (2001), Pineapple: Borrás et al. (2001)).Utilization of culture filtrate as a selective agent has better selectivity compared to use of pathogenic toxin alone. Apart from toxins, culture filtrate contains many secondary metabolites (polysaccharides, oligosaccharides (Peros and Chagvardieff, 1987), proteins, glycoproteins, unsaturated fatty acids, stem from the cell walls, cytoplasm of the pathogen, growth regulators such as auxin, kinetin and gibberellic acid (Gentile *et al*., 1992)) which are important as co-determinants of the plant-pathogen interaction and disease development (Buiatti and Ingram, 1991; Crino, 1997; Švábová and Lebeda, 2005).

The kind of toxins and other substances present in the filtrate are depended on the disease and the pathogen.

Table 1.3: *In vitro* selections for disease resistace in crop plants (Source:Review;Penna et al., 2012)

Crops	Selective agent	Resistance	Reference
Annona comosus	Filtrate, FA	*Fusarium subglutinas*	Borras *et al.* 2001
Arachis hypogaea	CF	*Cercosporidium personatum*	Venkatachalam and Jayabalan 1996
Brassica napus	CF	*Phoma lingam*	Sacristan 1982
	Partially purified CF	*Alternaria brassicicola*	MacDonald and Ingram 1985, 1986
Carica papaya	Sporangial suspension of *Phytophthora palmivora*	*Phytophthora* wilt	Sharma and Skidmore 1988
Carthamus tinctorius	CF	*Alternaria carthami*	Kumar *et al.* 2008a
Citrus limon	CF	*Phoma tracheiphila*	Gentile *et al.* 1992
Curcuma	CF	*Pythium graminicolum*	Gayathri *et al.* 2005
Curcuma longa	CF	*Pythium graminicolum*	Gayatri *et al.* 2005
Fragaria vesca	Partially purified toxins	*Phytopthora cactorum, Rhizoctonia fragariae, Botrytis cineria*	Battistini and Rosasti 1991; Orlando *et al.* 1997; Remoti 1998
Gladiolus grandiflorus	FA	*Fusarium oxysporum*	Remotti *et al.* 1997
Glycine max	CF	*Septoria glycines*	Song *et al.* 1994
Gossypium hirsutum	CF	*Fusarium oxysporum, Alternaria macrospora*	Ganesan and Jayabalan 2006
Hordeum vulgare	FA	*Fusarium* spp.	Chawla and Wenzel 1987
Linum usitatissimum	CF	*Fusarium oxysporum*	Krause *et al.* 2003
Lycopersicon esculentum	CF	*Pyrenochaeta lycopersici*	Fujime and Fujime 2003
Malus domestica	CF	*Phytopthora cactorum*	Utkhede 1986
	Co-cultivation	*Venturia enequalis*	Raman and Goodwin 2001
Mangifera indica	toxin	*Colletotrichum gloeosporioides*	Jaysankar *et al.* 1999
Medicago sativa	CF	*Fusarium oxysporum* f.sp. *medicaginis*	Hartman *et al.* 1984; McCoy 1988
Musa spp.	CF	*Fusarium oxysporum*	Matsumoto *et al.* 1999
Oryza sativa	CF	*Helminthosporium oryzae*	Vidhyasekaran *et al.* 1990
Peach	Fractionated CF	*Xanthomonas campestris* pv. *pruni*	Hammerschlag 1988
Psidium guajava	Cell-free filtrate	*Penicillium vermosonii wilt*	Vos *et al.* 1998
	CF of *F. oxysporum* sp. *solani*	*Fusarium oxysporum wilt*	Bajpai *et al.* 2007
Solanum tuberosum	CF	*Phytophthora infestans*	Behnke 1980
Tobacco	Methionine sulfoximine	*Pseudomonas syringae*	Carlson 1973
Triticum aestivum	DON	*Fusarium* sp.; *Fusarium graminearum*	Maier and Oettler 1992; Yang *et al.* 1998
Saccharum officinarum	Phyotoxin	*Colletotrichum falcatum*	Mohanraj *et al.* 2003
	Toxin	*Helminthosporium sacchari*	Heinz *et al.* 1977; Larkin and Scowcroft 1983
	CF	*Colletotrichum falcatum*	Sengar *et al.* 2009
Vitis vinifera	Dual culture	*Plasmopora viticola*	Barlass *et al.* 1986
	Filtrate	*Bortrytis cinerea*	Reustle and Matt 2000
	CF	*Elsinoe ampelina*	Jayasankar *et al.* 2000
Wheat	Syringomycin	*Pseudomonas syringae* pv. *syringae*	Pauly *et al.* 1987
Zea mays	HmT toxin	*Helminthosporium maydis*	Gengenbach *et al.* 1977

Slavov and Blagoeva-Nikolaeva (1995) showed that the nontoxic compounds present in the culture filtrate can show positive or negative effects on the surviving and regeneration of *in vitro* tissue cultures. In the study of Varidi et al., (1986) for resistance screening in *Citrus* against *Phytophthora citrophthora,* couldn't use culture filtrate due to presence of indole acetic acid in the filtrate and it was negatively affected on the regeneration. Sometimes, secondary metabolites present in the filtrate may show phytotoxic effect on the plant materials but without any involvement with disease development (Yoders, 1983). There are many studies reported that have been done to evaluate the effects of substances contained in the selected culture filtrates and fond those compounds in various filtrates show host-selective and nonhost-

selective plant-pathogen interactions (Lebeda and Švábová, 2010). Those facilitate the elicitation of different defense responses and metabolic reactions against disease development. For examples, activation and production of some enzymes (i.e. phytoalexin, peroxidases, β 1,3-glucanase, chitinase) (Saindrenan *et al*., 1990; Crino, 1997; Lebeda *et al*., 2001; Jayasankar and Litz, 1998; Lebeda *et al*., 2001; Singh *et al*., 2003), accumulation of phenolic acids and phenols (Cvikrová *et al*., 1992).

1.2.10 *In vitro* mutagenesis in *Brassica*

As discussed previously, *in vitro* mutations create novel and unique variations (Chopra and Sharma, 1991) and has become as an important technique in mutation breeding preventing or restricting chimera formation (Reviewed in Velmurugan et al., 2010). *In vitro* mutagenesis and selection have been successfully applied to different *Brassica* varieties to develop or improve various characters. Dunemann and Grunewaldt (1990) developed dominant mutation for male sterility in broccoli (B. oleracea var. ifalica) treating inflorescence explants with N-nitroso-N-methyl-urea(NMU) and Feng-Ian and Takahata (1999) has developed soft rot disease resistance in chinese cabbage (*B. campestris* ssp. *Pekinensis)* by UV irradiation mutation induction in microspore and *in vitro* selection using bacterial culture filtrate. It has also developed cauliflower (*B. oleracea* var. *botrytis*) carrying drought and salt stress tolerance by chemical mutagenic treatment of N-nitroso-N-methylurea (NEU) and NMU (Haid and Fuller, 2013) and resistance to black rot pathogen *Xanthomonas campestris* pv. *Campestris* using ethyl methansulfonate (EMS) and gamma radiation (Mangal and Sharma, 2003). Ali and Ahamad (2004) analyzed the mutation induction and in vitro embryogenesis applying chemical mutagenesis treatment (EMS) to microspore of *B.napu*. Yun et al. (2005) also discussed about mutagenic effect of UV radiation, EMS and NaN_3 on in vitro plant regeneration from cotyledon explants of *B. napus*. In addition to advantageous mutation induction, some adverse effects of mutagenic treatments, low mutation rates, random mutations and tremendous works of assessments in offspring and toxic residues of the mutagens can be observed in in vitro mutagenesis (An et al., 2003).

CHAPTER 02

Materials and Method

CHAPTER 02

Materials and Methodology

2.1 Materials

2.1.1 Biological materials

2.1.1.1 *Brassica* seeds

Brassica oleracea L. var. capitata L. seeds of six cultivars; Pourovo pozdani (PP), Kiklop F1 (KI), Zeus F1 (ZF), DC 6 (DC), Target F1 (Ta) and Albastros F1 (AL) were obtained from the seed company; Moravo seeds CZ a.s in Czech Republic.

2.1.1.2 *Fusarium oxysporium f.* sp. *Conglutinans strain*

Isolated *Fusarium oxysporium f.* sp. *Conglutinans* strain 1 was obtained from Crop Research Institute in Prague, Czech Republic.

2.1.2 Chemicals

All chemicals used during the study (of molecular grade) were obtained from one of the following sources.

Sigma Chemical Co, St.Louis, USA.

Carl Roth GmbH Co.Kg

ŠK Spektrum S. R. O . Slovakiya.

2.1.2.1 For media preparation

The list of chemicals, which was used for the media preparation, are given in the appendix.

2.1.2.2 For seed surface sterilization

Ethanol (70%), Clorox (20%), Tween twenty

2.1.2.3 For mutagenesis treatment

Dimethylsulphoxide (DMSO)

Ethyl methanesulfonate (EMS)

2.1.3 Growth regulators

Napthalene acetic acid (NAA),

6- benzylaminopurine (BAP),

Indole-3-butyric acid (IBA)

Gibberellic Acid (GA3)

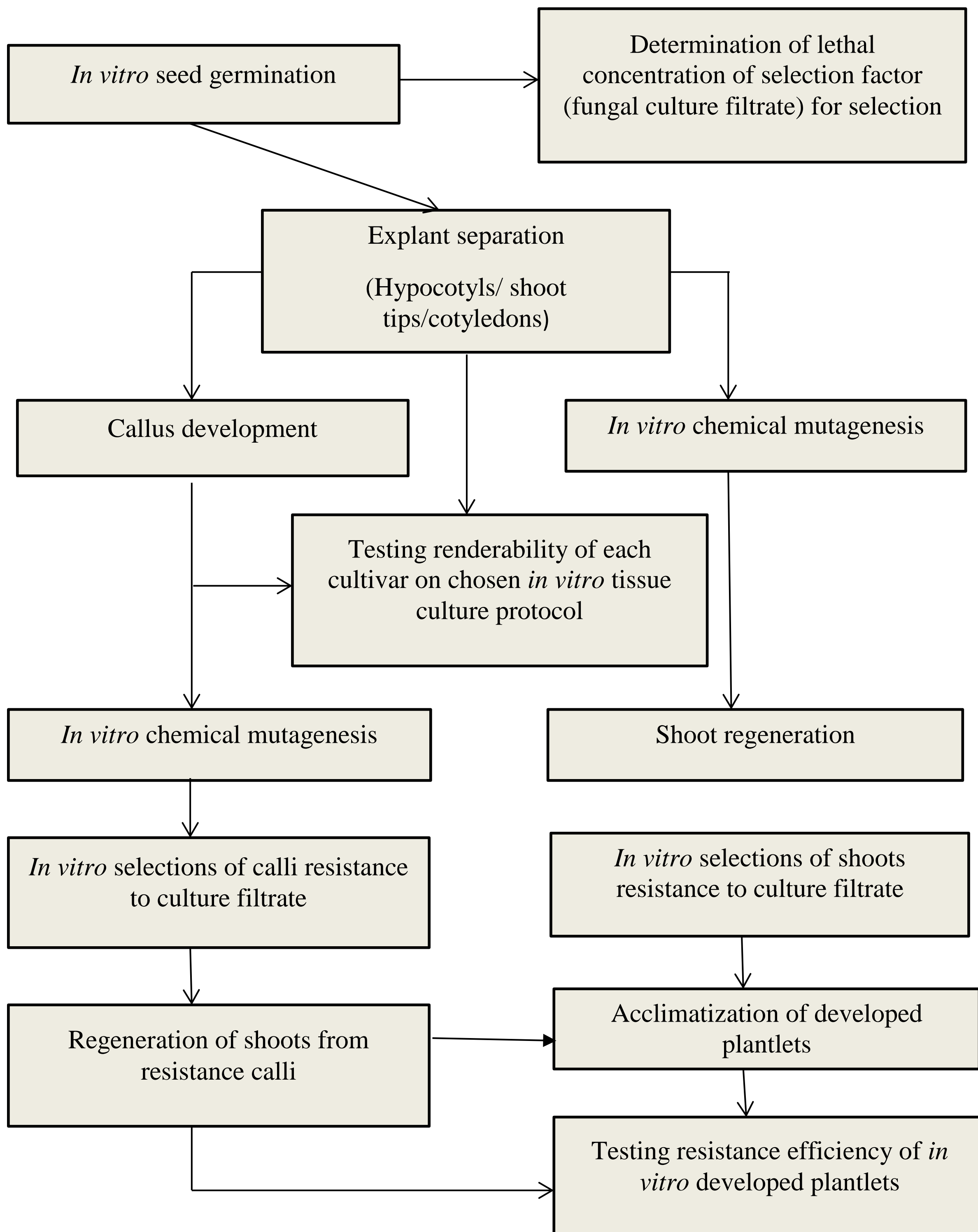
2.1.4 Equipments used for the media preparation, tissue culture and mutagenesis processes

All the following equipments used for media preparation, tissue culture and mutagenesis treatment were obtained from Department of Horticulture, Czech University of Life Sciences in Prague, Czech Republic.

- Balance (SCEOO001, Scaltec instruments, Germany)
- pH meter (Greisinger Electronics, Germany)
- Autoclave (MLS3781L/ SANYO electronics co. Ltd, Japan)
- Laminar flow hood (FlowFAST H 18, Italy)
- Centrifuge machine (HiTEC/ Universal 320R, Germany)
- Micro filters 0.22 µm (Watman, United Kingdom)
- Micro pipets

2.2 Methodology

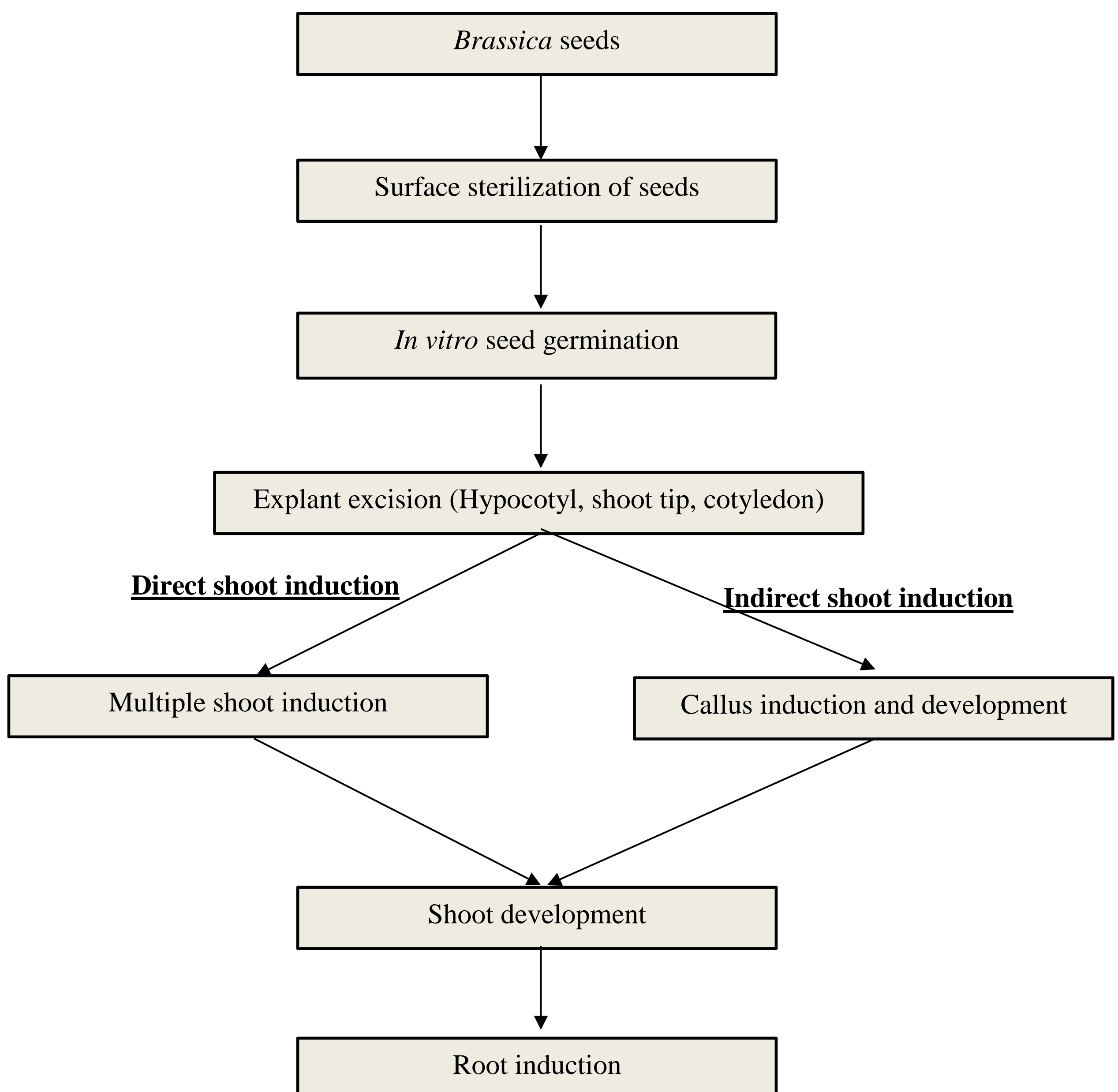
2.2.1 Summary of the study, developing *Fusarium* Resistant *Brassica oleracea L. var. capitata L.* plants through *in vitro* chemical mutation induction.



2.2.2 Development of cabbage plantlets through *in vitro* plant tissue culture

To determine the regeneration capacity (without subjecting to *in vitro* mutagenesis) in chosen cabbage tissue culture protocol, direct and indirect shoot regeneration from three different explants excised from 6 days and 10 days old *in vitro* germinated seedlings were carried out as the basic procedure mentioned below per Bhalla and Smith (1998) and Bhalla and Weerd (1999).

2.2.2.1 Summary



2.2.2.2 Media preparation and glass ware sterilization

All the media were prepared per the compositions as mentioned in appendixes and those were autoclaved at 121 °C for 20 minutes at 15psi pressure for sterilization.

Sterilized plastic petri plates were used in this study and all other glass wares used under aseptic condition throughout the study were sterilized as same as in media preparation.

2.2.2.3 Direct and indirect shoot regeneration

2.2.2.3.1 Surface sterilization of seeds

Cabbage seeds were subjected to surface sterilization procedure to eliminate the microorganisms which are on contact with seed surface, minimizing the occurrence of the seed born contaminations in *in vitro* culture conditions.

First, seeds were washed with 70% ethanol for 1 minute and then with 20% Clorox (NaOCl) with 2-3 drops of Tween twenty for 20 minutes. Finally, seeds were washed 3 times with sterilized distilled water to remove chemicals used previously and they were blot dried using sterilized filter papers.

All these steps were carried out under fully aseptic conditions (in laminar flow cabinet).

2.2.2.3.2 *In vitro* seed germination

After subjecting to surface sterilization process, seeds were placed on MS seed germination medium (Appendix A) which contains MS medium (Murashige and Skoog, 1962) supplemented with Sucrose (10 g/L), phytagel (7g/L) in pH 5.8 and cultures were incubated in light at 25 ºC ± 2 ºC for 6 to 10 days.

2.2.2.3.3 Direct shoot induction

Three different explants; hypocotyl, shoot tip and cotyledons were excised from 6 days and 10 days old *in vitro* derived seedlings of all six cultivars and were cultured separately on petri plates with MS shoot regeneration medium (15 mL) (Appendix B) supplemented with Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), $AgNO_3$ (0.5 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated for three weeks at 25 ºC ± 2 ºC in light.

After each three weeks' cultures were transferred repeatedly to fresh same regeneration medium and incubated at same culture conditions until developing clear small shoots with approximately 0.5 cm height.
Data were collected after each week.

2.2.2.3.4 Indirect shoot induction

Shoots were induced through calli derived from two different explants (hypocotyl and cotyledons) excised from 6 and 10 days old *in vitro* germinated seedlings.

2.2.2.3.4.1 Callus induction

Hypocotyl and cotyledon explants excised from 6 days and 10 days old *in vitro* derived seedlings were cultured separately on petri plates with MS callus induction medium (15 mL) (Appendix C) supplemented with Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), $AgNO_3$ (0.5 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated for several weeks at 25 ºC ± 2 ºC in light.
Data were collected after each week.

2.2.2.3.4.2 Shoot induction from calli

After two to three weeks on callus induction medium, callus were transferred to MS shoot induction medium (Appendix B) supplemented with Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), $AgNO_3$ (0.5 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated at 25 ºC ± 2 ºC in light.
In each two week, growing callus with inducing shoots repeatedly were transferred to fresh same MS shoot induction medium in each 14 day and incubated at same culture conditions until developing clear small shoots with approximately 0.5 cm height.
Data were collected after each week.

2.2.2.3.5 Shoot development

For the purpose of achieving shoots with well grown and sufficient enough height, shoots derived from both direct and indirect shoot induction protocol were transferred to MS shoot development medium (Appendix D) supplemented with Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3

mg/L), GA_3 (0.01 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated for 15 to 30 days at 25 ºC ± 2 ºC in light till they reach at least 2 cm height.

2.2.2.3.6 Regeneration of roots

After sufficient shoot elongation, these shoots were transferred to MS rooting medium (Appendix E) supplemented with IBA (0.2 mg/L), Sucrose (10 g/L), and phytagel (7 g/L) in pH 5.8 and incubated at 25 ± 2 ºC in light for few weeks (maximum 8 weeks).

2.2.3 Development of *Fusarium* resistance cabbage plants

Fusarium resistant cabbage plants of all six cultivars used were developed following *in vitro* mutation induction and selection.

2.2.3.1 Preparation of *Fusarium* fungus culture filtrate

A small amount (2mm x 2mm) of isolated fungal colony (Figure 2.1) is inoculated into Czapek-Dox liquid medium (150 mL/ From the dehydrated pre prepared product (SIGMA), 35 g/L) in pH 6.6. The culture was incubated at 28 ± 2°C for 21 days in light, without shaking. The cultured liquid medium was then filtered through a filter paper and Centrifuged at 8000 rpm for 20 minutes to precipitate mycelium and conidia. The pH of the supernatant was adjusted to 5.8 and filter sterilized using membrane with 0.22 µm pore size. This fungus-free culture medium was used as the selection agent for resistance screening from mutated plant cultures.

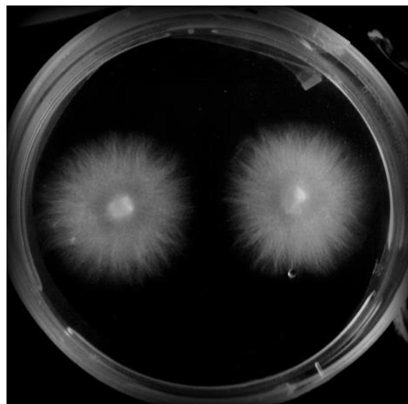


Figure 2.1: Isolated *Fusarium oxysporium f.* Sp. *Conglutinans* on Czapek-Dox solid medium.

2.2.3.2 Determination of optimum pathogenic concentration of culture filtrate for selection

To determine the optimum concentration of fungus culture filtrate for select plants carrying *Fusarium* resistance mutations, *In vitro* germinated 10 days old seedlings were cultured into MS shoot development medium (Appendix D) supplemented with series of concentrations (5%, 10%, 15%, 20% and 25% (v/v)) of fungus culture filtrate and Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated for 30 days at 25 ºC ± 2 ºC in light. Control was carried out in the same condition without incorporation of fungus culture filtrate.

2.2.3.3 Application of chemical mutagenesis treatment

Plant materials; explants (hypocotyls and shoot tips) excised from 6 days and 10 days old seedlings and seven days old calli were incubated in aqueous solution supplemented with filter sterilized dimethylsulphoxide (DMSO) (4% (v/v)) and ethyl methanesulfonate (EMS) (0.3% (v/v)) for 2 hours at 28 ± 2°C. After washing three times with sterile distilled water following chemical treatment, plant materials were cultured either on MS shoot induction medium for several weeks or on MS callus induction medium for one week.

All these steps were carried out under fully aseptic conditions (in laminar flow cabinet).

2.2.3.4 Selection of calli and shoots carrying *Fusarium* resistance mutations

Following successful mutagenesis treatment calli were cultured into fresh same callus induction medium (Appendix C) exceptionally containing fungus culture filtrate (15% (v/v)) and incubated for 30 days at 25 ºC ± 2 ºC in light.

After 30 days, surviving calli were transferred to same callus induction medium containing (20% (v/v)) and incubated for 30 days at 25 ºC ± 2 ºC in light. Surviving calli were subjected to further selection under same fungus culture filtrate (20% (v/v)) for another 30 days at 25 ºC ± 2 ºC in light and then shoots were developed form selected surviving calli through shoot induction and development as same as described above in 2.2.1.3.4.2 and 2.2.1.3.5.

Same as for treated calli, regenerated shoots with sufficient height (≈1.5 cm) following successful mutagenesis treatment and shoot induction, were incubated in MS shoot

development medium consisting fungus culture filtrate (15% (v/v)) for 30 days at 25 ºC ± 2 ºC in light and another two repeats of 30 days period in the medium containing fungus culture filtrate concentration of 20% (v/v) at 25 ºC ± 2 ºC in light. Surviving shoots were transferred into root induction medium.

2.2.4 Acclimatization of in vitro derived plants

Properly rooted plantlets, at least 3-4 number of roots with more than 2cm long were transferred into sterilized substrate (profit mix from Agro cs a.s. company) and gradually trained from 100% humidity and elevated temperature to normal environmental conditions. Data of successfully adapted plants were collected after 45- 60 days.

The data were subjected to analysis of variance (factorial ANOVA). The means were compared by Tukey's test ($P < 0.05$).

CHAPTER 03

Results

Chapter 03

Results

3.1 *In vitro* seed germination and explant excision

Following the successful surface sterilization and germination induction on the MS seed induction medium (appendix A), germination of seeds of all six cultivars were initiated within 24 hours. Four cultivars; PP, DC, Ta and AL were showed 100% germination rate by the day 7 after germination induction while ZF and Ki having 94.0% and 44.0% germination rate respectively. Six days old seedlings were reached 2.5 cm – 3.5 cm height and by the 10 days they were reached to 4.0 cm – 5.5 cm height with cultivar Ki was showing shorter seedlings while cultivar PP having highest seedlings.

Three different explants; hypocotyls, shoot tips and cotyledons were excised form six and ten days old seedlings (Figure 3.1) and cultured either on MS shoot induction medium (Appendix B) or MS callus induction medium (Appendix C) depending on which organogenesis pathway (direct/indirect) followed.

Figure 3.1: Seed germination and explant excision. a.) Seeds on the MS germination medium (day 0). b.) 6days old seedlings. c.) Hypocotyl explants on MS regeneration induction medium. d.) Shoot tip explants on MS regeneration induction medium. e.) Cotyledons explants on MS regeneration induction medium. All explants were from 6days old seedlings. Bar = 10mm

3.2 Determination of regeneration efficiency

The regeneration efficiency of selected six cultivars of *Brassica oleracea L. var. capitata L.* through direct and indirect shoot induction by chosen *in vitro* culture protocols (as described by Bhalla and Smith (1998) and Bhalla and Weerd (1999)), and from three different explants (hypocotyl, shoot tip, cotyledon) obtained from two different old seedlings (6 days and 10 days) were tested before under go *in vitro* mutagenesis and resistance selection against *Fusarium.*

3.2.1 Direct shoot induction and development

Explants excised from *in vitro* germinated seedlings with the age 6 days and 10 days, were cultured on MS shoot induction medium (Appendix B) and incubated at 25 ^{0}C ± 2 ^{0}C under light.

Within first week on the regeneration medium, pale yellow color hypocotyl explants were turned into greenish and swollen while shoot tip explants were increased its volume both length and width growing its main shoot tip and cotyledon explants were enlarging its size approximately three times of its starting size. Within 21 days on same culture medium, multiple shoot induction was appeared at distal ends of the hypocotyl explants of all six cultivars and by the 30th day clear multiple shoot induction was observed (Figure 3.3).

In some hypocotyl explants were showed slight formation of calli at the basal ends. But comparing all six cultivars, ZF, PP and Ta were showed better performance with clear multiple shoot induction by the 30th day on shoot induction medium than cultivars Ki, DC and Al which showed small shoots on the explants. By the day 45 to 60 days, there were clear shoot growth from both 6 days and 10 days old hypocotyls of all cultivars. Table 3.1 shows the percentage of expalnts inducing shoots and number of shoots per explants produced by each three types of explants in each age groups.

Generally, by the 14 – 21 days, shoot tip explants were started to induce multiple shoots at the base of shoot tip (Figure 3.3) and within 45 days clear multiplication of shoots were observed. But, different cultivars were showed different responses on multiple shoot induction by both 6 days and 10 days old shoot tip explants. Cultivars, PP, ZF showed highest multiple shoot induction from both 6 days and 10 days old shoot tip explants with 86.7%/ 92% for PP and 72%/ 80% for ZF respectively from 6 days and 10 days old explants. There was not multiple shoot induction from 10 days old shoot tip explants of cultivar Al and less than 50% multiple shoot induction was observed in cultivars DC, KI and Ta from both ages shoot tip explants

(Table 3.1 and Figure 3.2). As in the hypocotyl explants, here also the number of transferable shoots developed were less compared to percentage of shoot induction.

Both aged cotyledon explants in all cultivars showed very low multiple shoot induction and clear development (Figure 3.3). The cultivars DC didn't show any shoot induction in neither 6 days old nor 10 days old except cultivars, Ta and Al were giving same response only in 10 days old cotyledons. During this study, with the time cotyledon explants were showed yellowing and turning into black leading to death. This chlorosis and necrosis were a problem for organogenesis.

According to statistical analysis, shoot induction and number of shoots per explant producing by each three different explant types in each age groups were significantly different among cultivars, among explant types and among age of the explant (Figure 3.2). Different cultivars showed different responses along each type of explants and age of the explants. Compared to both hypocotyl and shoot tip explants, cotyledon showed the lowest shoot production in both 6 and 10 old ages. Comparatively in general, 6 days old hypocotyl explants showed lower percentage of shoot induction (maximum 82% in ZF and minimum 43.3% in Ta) than 10 days old hypocotyls (maximum 100% in PP and minimum 22% in DC). But, 6 days old hypocotyl showed higher production of transferable number of shoots per explant (maximum 5.2 ± 0.8 in PP and minimum 1.2 ± 0.2 in Ki) after 45 to 60 days on shoot induction medium than 10 days old hypocotyls (maximum 1.9 ± 0.6 in Ki and minimum 0.5 ± 0.2 in DC).
Depending on the observed and analyzed data, hypocotyl and shoot tip explants were selected for next step (mutagenesis) studies of this study.

Table 3.1: Average number of shoots per explant and percentage of total number of shoots developed by three different explants excises from 6 days and 10 days old seedlings of six cultivars after 45-60 days of culture initiation.

Cultivar		Explant					
		Hypocotyl		Shoot tip		Cotyledon	
		Explants with shoots (%)	Average no. of shoots/explant (±SD)	Explants with shoots (%)	Average no. of shoots/explant (±SD)	Explants with shoots (%)	Average no. of shoots/explant (±SD)
ZF	6 Days	82,0	2,8±0,5	72,0	5,9±0,6	30,0	1,3±0,5
PP		58,7	5,2±0,8	86,7	3,2±0,4	20,0	1,3±0,5
DC		47,3	2,5±0,2	32,0	1,2±0,1	0,0	0,0
Ki		52,7	1,2±0,2	20,0	0,6±0,6	5,0	2,0±1,6
Ta		43,3	2,0±0,4	8,0	0,0	1,7	0,3±0,4
AL		46,7	3,4±0,5	40,0	0,7±0,2	1,7	0,3±0,4
ZF	10 Days	98,0	1,6±0,2	80,0	3,1±0,5	15,0	1,3±0,3
PP		100,0	1,5±0,4	92,0	3,3±0,4	30,0	0,7±0,1
DC		22,0	0,5±0,2	24,0	0,9±0,2	0,0	0,0
Ki		52,7	1,9±0,6	40,0	1,3±0,7	1,7	0,3±0,5
Ta		56,0	0,9±0,1	16,0	0,5±0,4	0,0	0,0
AL		90,0	1,1±0,5	1,3	0,3±0,5	0,0	0,0

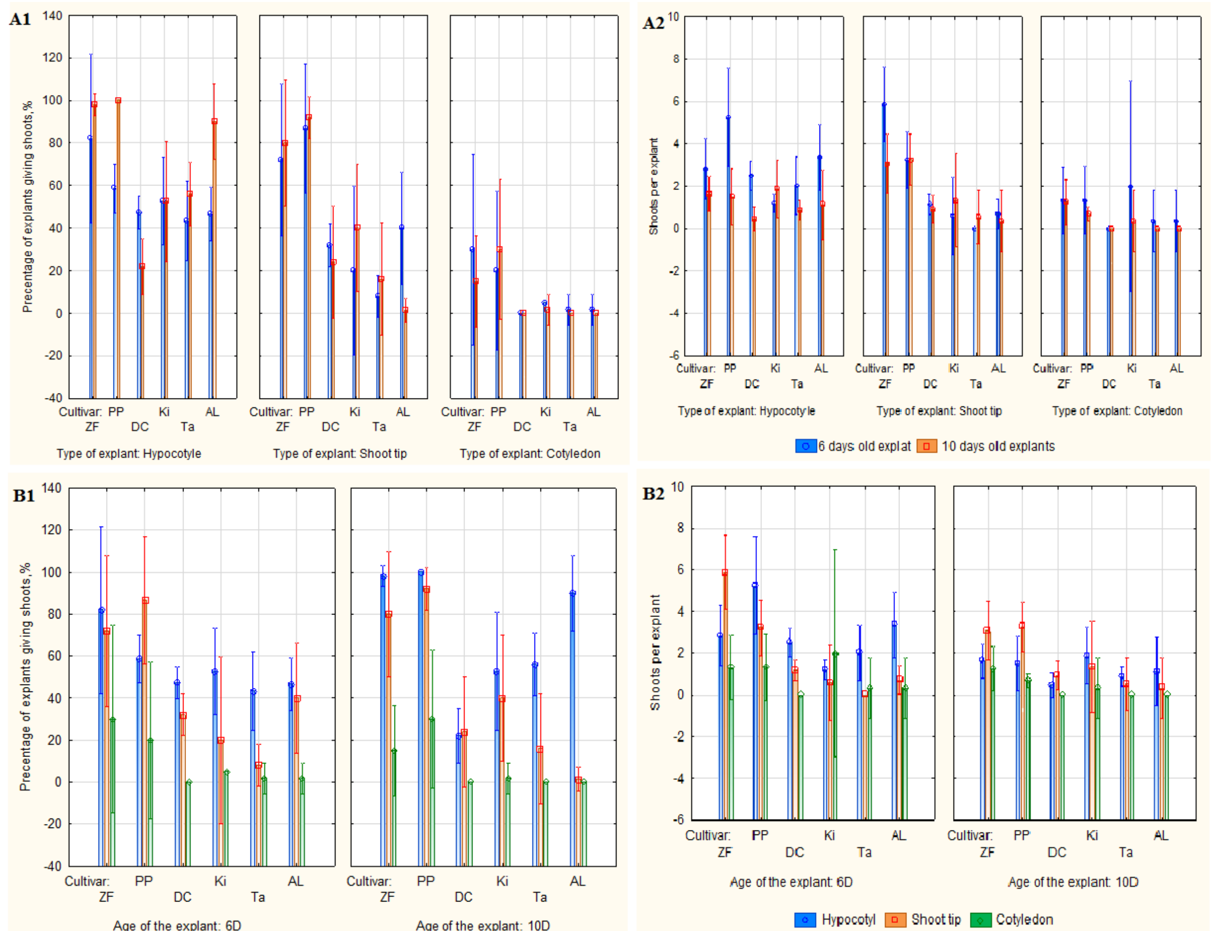


Figure 3.2: Different responses of shoot induction and number of shoots producing by different explants with two different age groups of six different cultivars through direct organogenesis without mutagenesis treatment.

A. Comparison between two age groups of explants by percentage number of explants inducing shoots and number of shoots per explants of different explants in each cultivar.

B. Comparison between different explants by percentage number of explants inducing shoots and number of shoots per explants of each cultivar in 6 and 10 days old age group.

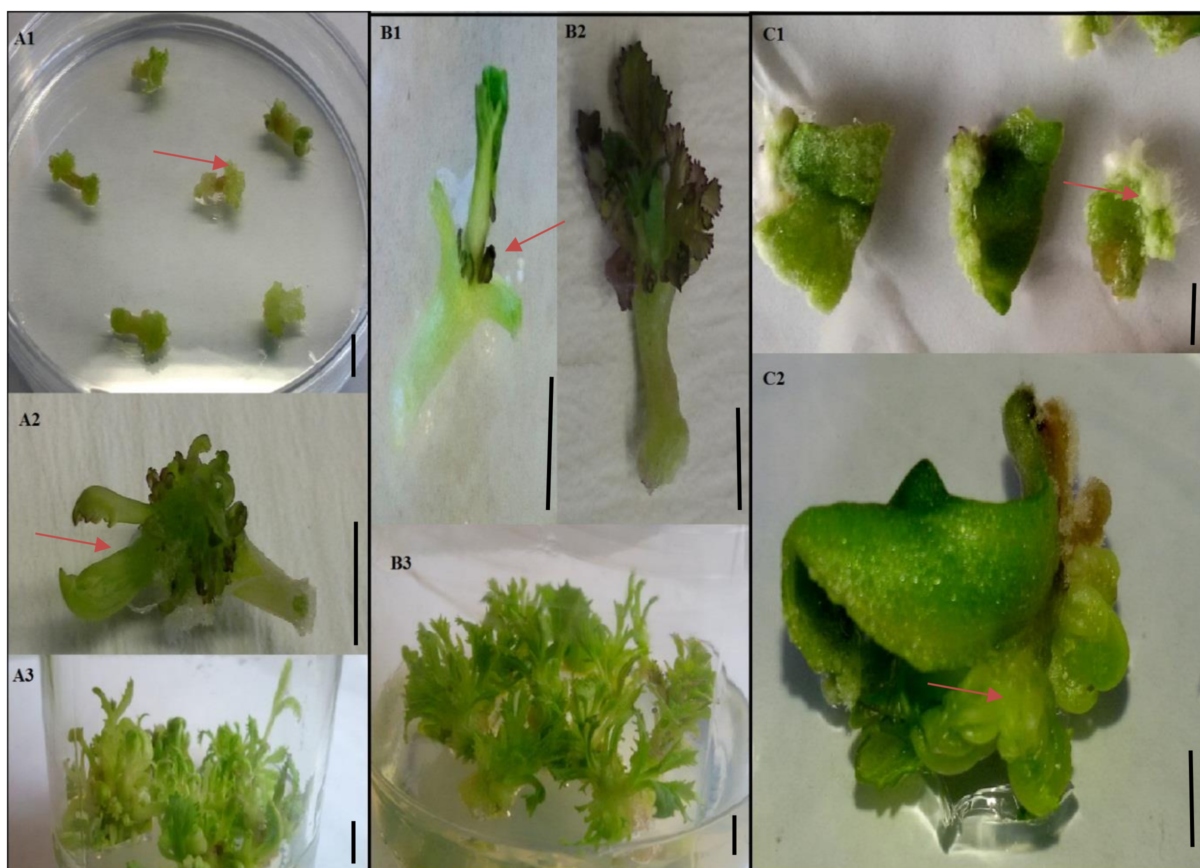


Figure 3.3: Direct shoot induction from explants excised from 6 days old seedlings without mutagenesis, on MS shoot regeneration medium (15 mL) (Appendix B) supplemented with Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), $AgNO_3$ (0.5 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated for three weeks at 25 ºC ± 2 ºC in light. Arrows are pointed to the inducing and developing shoots. Bar scale = 10 mm.

A. Hypocotyl explants; A1. Initiation of shoot induction after 14 days in MS shoot induction medium, A2. Clear shoot induction after 21 days, A3. Clear multiple shoot induction after 30-45 days.
B. Shoot tip explants; B1. Initiation of shoot induction after 14 days, B2. Clear multiple shoot induction after 21 days, B3. Clear multiple shoot development after 30 days.
C. Cotyledon explants; C1. Initiation of shoot induction and swollen explants after 14 days, C2. Multiple shoot induction at the cut edges of the cotyledon after 21- 30 days.

3.2.2 Indirect shoot induction and development

Explants (hypocotyl and cotyledons) excised from *in vitro* germinated seedlings with the age 6 days and 10 days, were cultured on MS shoot induction medium (Appendix B) and incubated at 25 ^{0}C ± 2 ^{0}C under light.

In general, both 6 days and 10 days old hypocotyl explants of all six cultivars showed yellowish callus induction within 14 days on callus induction medium with the percentage ranging from 46.0% to 95.3%. But 6 days old hypocotyl were showed better performances with more than 60.0% callus induction in all cultivars. It was ranged from 64.0% to 95.3% (Table 3.2 and Figure 3.4). There was no significant difference of callus induction between 6/10 days old hypocotyl explants or among each cultivar except cultivar Ki. Both 6 and 10 days old hypocotyl explant were given higher callus induction than cotyledon explants and it was significantly different.

Overall all six cultivars, the callus derived from both 6 days and 10 days old hypocotyl explants showed significantly similar multiple shoot induction ranging from 42.0% in “Ki” to 96.0% in “PP”. But, the generation of number clear shoots which is transferable was low and different among cultivars. Even though calli showed higher multiple shoot induction, the number of transferable shoots developed were low and maximum average number of shoots per explant was around 2. Calli derived from 6 days old hypocotyl had higher number of shoots development per explant (maximum 1.9±0.1) than from calli derived from 10 days old hypocotyl explant (maximum 0.8±0.1).

Interestingly in all cases six days old explants were given better percentage of shoot induction and better production of shoots per explants in all cultivars and between two types of explants; hypocotyl and cotyledon, hypocotyl explants showed better performances in indirect organogenesis for selected *Brassica* cultivars. Therefore, depending on all analysis six days old hypocotyl explant was selected as the starting material for next step of the study through indirect organogenesis.

Table 3.2: Average number of shoots per calli and percentage of total number of shoots developed by two different explants excises from 6 days and 10 days old seedlings of six cultivars after 45-60 days of culture initiation.

Cultivar		Explant			
		Hypocotyl		Cotyledon	
		Calli with shoots (%)	Average no. of shoots/callus (±SD)	Explants with shoots (%)	Average no. of shoots/callus (±SD)
ZF	6 Days	90,0	1,7±0,2	40,0	1,3±0,5
PP		96,0	1,9±0,1	55,0	1,6±0,8
DC		52,0	1,4±0,3	25,0	0,7±0,5
Ki		48,0	1,2±0,2	35,0	0,2±0,2
Ta		78,0	1,3±0,2	30,0	1,3±0,4
AL		54,0	1,2±0,1	20,0	0,9±0,3
ZF	10 Days	92,0	0,8±0,1	90,0	1,3±0,3
PP		94,0	0,9±0,1	65,0	1,3±0,2
DC		50,0	0,5±0,1	0,0	0,0
Ki		42,0	0,8±0,1	35,0	0,0
Ta		76,0	0,7±0,1	20,0	0,0
AL		70,0	0,4±0,1	1,7	0,0

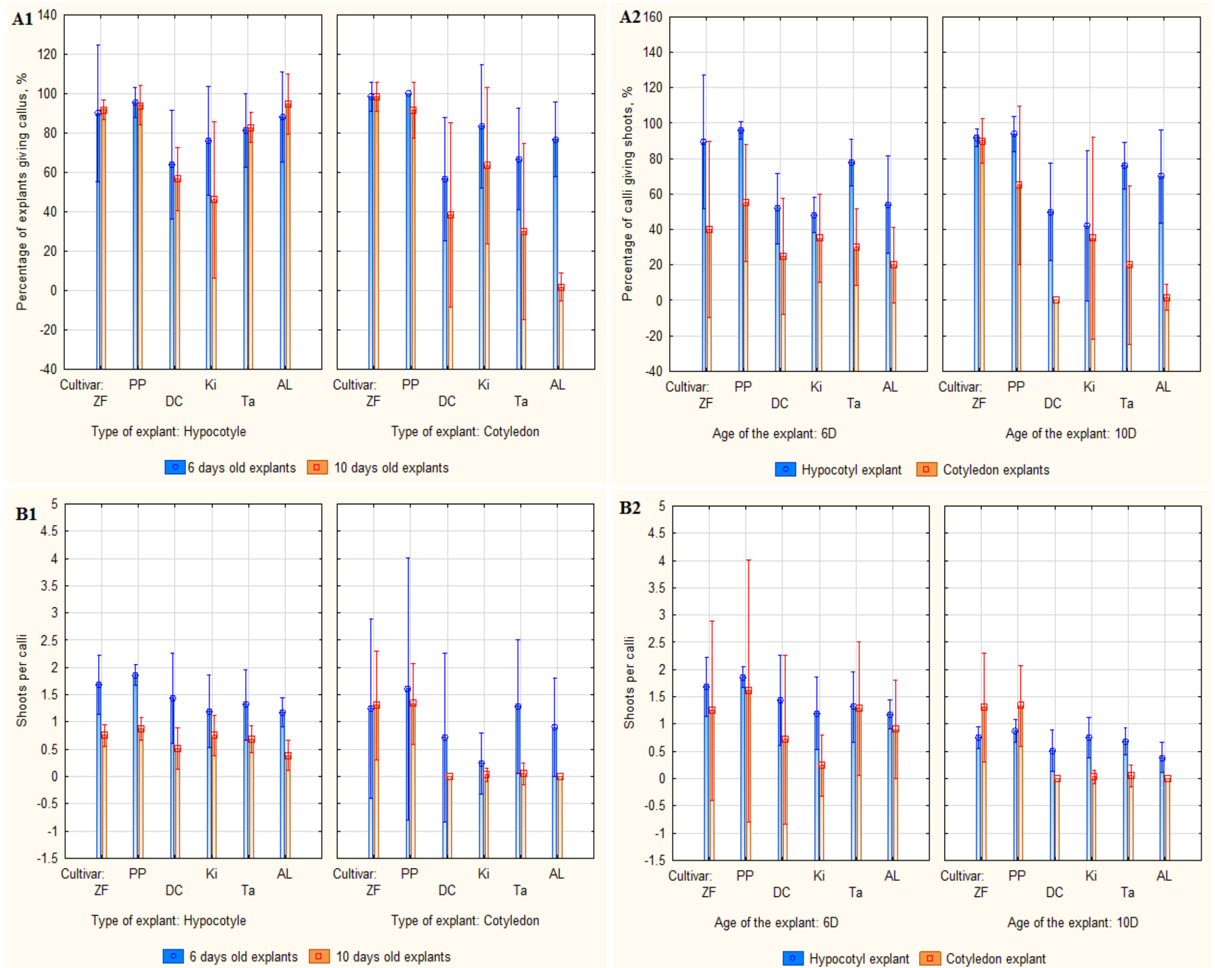


Figure 3.4: Different responses of shoot induction and number of shoots producing by different explants with two different age groups of six different cultivars through indirect organogenesis without mutagenesis treatment.

A1 and B1. Comparison between two age groups of explants by percentage number of explants inducing callus and number of shoots per calli of different explants in each cultivar.

A2 and B2. Comparison between different explants by percentage number of calli inducing shoots and number of shoots per calli of each cultivar in 6 and 10 days old age group.

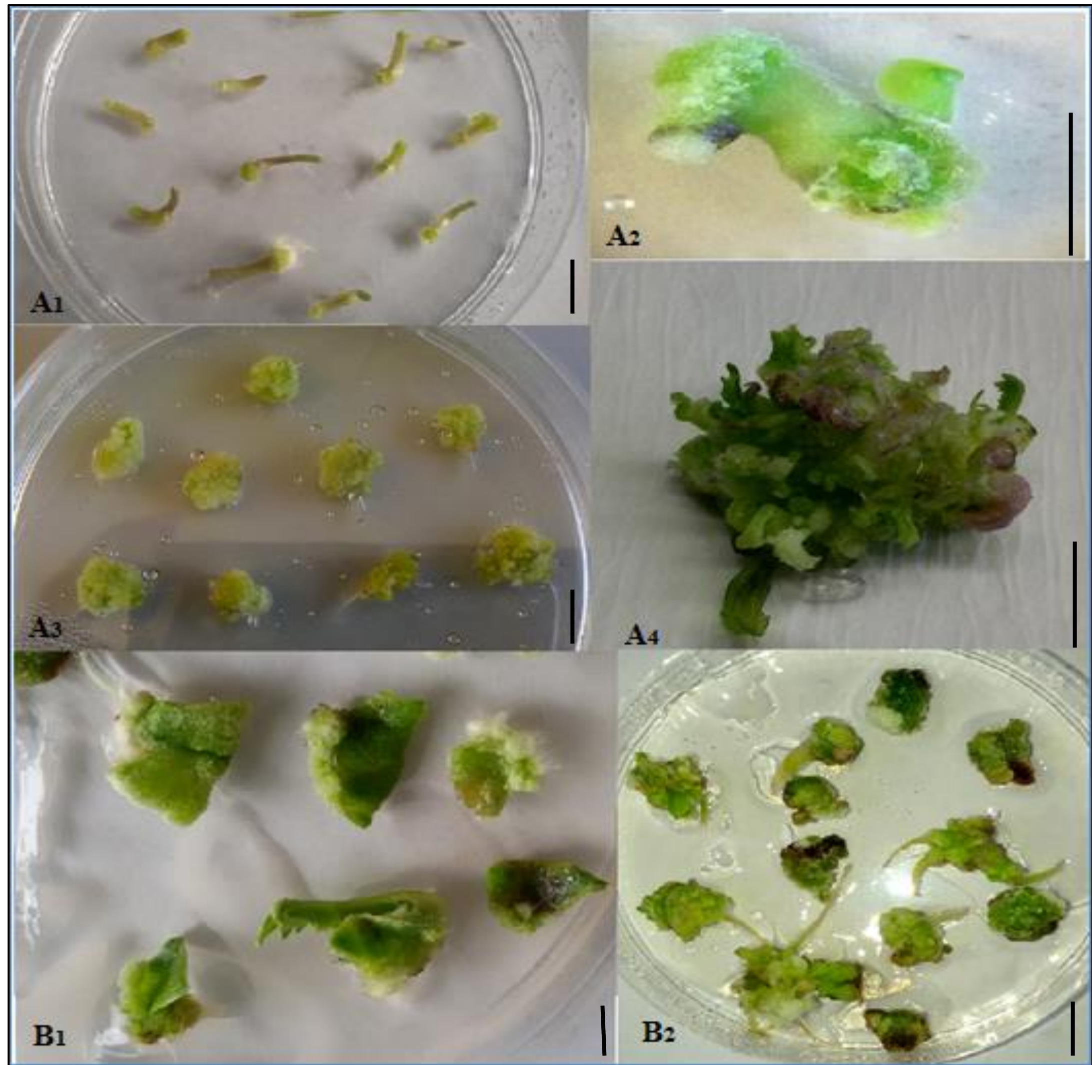


Figure 3.5: Indirect shoot induction from explants excised from 6 days old seedlings without mutagenesis, Bar scale = 10 mm.

A. Hypocotyl explants; A1. Initiation of callus induction after 10 days in MS callus induction medium (Appendix C), A2. Hypocotyl explant with clear callus induction at distal ends after 14 days. A3. Yellowish green calli on MS shoot induction medium (Appendix B) at day zero. A4. Callus with multiple shoot induction after 14 days on shoot induction medium.
B. Cotyledon explants; B1. Initiation of callus induction at the cut edges of the cotyledon and swollen explants after 14 days on Callus induction medium, B2. Developing calli without shoot induction, but inducing root like structures on shoot induction medium after 30 days.

In both direct and indirect organogenesis from cotyledon explants, chlorosis and necrosis were observed with the increase of the duration of the culture. Vitrification of developing shoots also observed in a considerable level.

3.2.3 Root induction

After sufficient shoot elongation, at least with 3.0 cm height, these shoots were transferred to MS rooting medium (Appendix E) and incubated at 25 ± 2 ºC in light for few weeks (maximum 8 weeks).

Figure 3.6: Root induction of developed shoots and acclimatization of rooted plantlets in to natural environment. Bar = 1 cm

A. Inducing roots in MS root induction medium (Appendix E) supplemented with IBA (0.2 mg/L), Sucrose (10 g/L), and phytagel (7 g/L) in pH 5.8 and incubated at 25 ± 2 ºC after 14 days.
B. Fully developed plantlet which is ready to acclimatize in to natural environment.
C. An acclimatized healthy plant.

Table 3.3: Percentage of root induction of *in vitro* produced shoots from different explants through direct and indirect organogenesis and acclimatization percentage of rooted plantlets of each cultivar.

		Explant					
		Hypocotyl		Shoot tip		Calli (hypocotyl)	
Cultivar	Age of the explants	Percentage of root induction (after 30-45days), %	Percentage of acclimatization. %	Percentage of root induction (after 30-45days), %	Percentage of acclimatization. %	Percentage of root induction (after 30-45days), %	Percentage of acclimatization. %
ZF	6 Days	96,7	48,3	80,0	70,8	70,0	61,9
PP		73,3	63,6	86,7	69,2	80,0	41,7
DC		43,3	38,5	36,7	18,2	20,0	16,7
Ki		70,0	57,1	20,0	50,0	3,3	40,0
Ta		53,3	50,0	0,0	0,0	56,7	2,5
AL		46,7	35,7	0,0	0.0	46,7	21,4
ZF	10 Days	80,0	70,8	83,3	56,0	-	-
PP		83,3	80,0	80,0	62,5	-	-
DC		10,0	0,0	13,3	25,0	-	-
Ki		46,7	50,0	40,0	25,0	-	-
Ta		53,3	37,5	16,7	40,0	-	-
AL		56,7	41,2	0,0	0,0	-	-

Some cultivars (ZF, PP) showed roots induction within 14 days in MS root induction medium supplemented with IBA (0.2 mg/L) and achieved 2.5 - 4.0 cm long roots in all cultivars except DC, Ta and AL by the 30th day in the medium. DC showed root induction by the 45th day but were few and shorter than other cultivars. Hypocotyl derived shoots of all six cultivars were showed more than 40% root induction (Table 3.3) while cultivar ZF showing maximum response in both age groups (96.7% and 80.0% respectively 6 and 10 days old hypocotyl) and DC showed the lowest root induction (43.3% and 10.0% respectively 6 and 10 days old hypocotyl. Maximum root induction of shoots derived from shoot tip explants was showed in cultivar PP (86.7%) under 6 days old age group while ZF having maximum percentage under 10 days old group. But some cultivars (Ta, Al) were showed zero root induction after 45 days

in root induction medium. Shoots derived from callus of cultivar Ki showed the lowest root induction (3.3%) while cultivar PP having 80.0% root induction. Acclimatization of in *vitro* developed plantlets were varies among cultivars. Plantlets derived from 10 days old hypocotyl of cultivar PP showed maximum acclamation rate (80.0%) and lowest in DC (0.0%).

3.3 Development of *Fusarium* resistance cabbage plants

In vitro chemical mutagenesis and selection were carried out to develop *Fusarium* resistant cabbage plants of all six cultivars selected for this study, through both direct and indirect organogenesis path ways.

3.3.1 Determination of optimum pathogenic concentration of fungus culture filtrate

In this study, *Fusarium* fungus culture filtrate was used as the selection agent for the selection of plantlets carrying *in vitro* chemically induced *Fusarium* resistant traits. For this purpose, to determine the optimal selection pressure, which cause phytotoxic effect on at least the 50% of the population.

The fungus culture filtrate was prepared by culturing isolated *Fusarium oxysporium f.* Sp. *conglutinans* in Czapek-Dox liquid medium pH 6.6 at 28 ± 2°C for 21 days in light, and finally subjecting to filter sterilization.

To assess the optimum toxic concentration with 50% effect on the plant population, *in vitro* germinated 10 days old seedlings were cultured into MS shoot development medium (Appendix D) supplemented with series of concentrations (5%, 10%, 15%, 20% and 25% (v/v)) of fungus culture filtrate and incubated for 30 days at 25 ºC ± 2 ºC in light. Control was carried out in the same condition without incorporation of fungus culture filtrate. Data was collected after 30 days which plants show toxic symptoms; yellowing, wilting or drying leaves, dead plants etc.

In 15 V/V% concentration, all six cultivars showed toxic characters; such as leaves were turning into yellow or brownish yellow and cut ends of the hypocotyls were becoming black (Figure 3.7). Cultivar DC showed more susceptibility with zero present survival at 15 V/V% concentration and cultivar Ki showed higher resistance with 60% survival rate at higher concentration (25 V/V%) while other cultivars were more or completely susceptible at the same concentration (Table 3.4 and Figure 3.7). Depending on the observed data, the concentration 15 and 20 V/V% were selected as the selection concentration for resistance screening.

Table 3.4: Phytotoxic effect of *Fusarium oxysporium f.* Sp. *conglutinans* culture filtrate on *in vitro* germinated *Brassica* seedlings.

Cultivar	Concentration V/V%	Survival percentage after 30 days (with or without symptoms) %	Symptoms observed
ZF	5	100,0	No observable symptoms
	10	100,0	No observable symptoms
	15	100,0	Yellow leaves and black cut end
	20	60,0	Yellow leaves and black cut end
	25	0,0	-
PP	5	100,0	Few yellow leaves with black cut ends
	10	80,0	Few yellow leaves with black cut ends
	15	50,0	Yellow leaves and black cut end
	20	20,0	Yellow leaves and black cut end
	25	0,0	-
DC	5	90,0	Few yellow leaves with black cut ends
	10	40,0	Yellow leaves and black cut end
	15	0,0	-
	20	0,0	-
	25	0,0	-
Ki	5	100,0	Green leaves / few black cut ends
	10	100,0	Green leaves / few black cut ends
	15	90,0	Yellow leaves and black cut end
	20	80,0	Yellow leaves and black cut end
	25	60,0	Yellow leaves and black cut end
Ta	5	100,0	small shaded yellow/ few black cut ends
	10	100,0	Few yellow leaves with black cut ends
	15	100,0	Yellow leaves and black cut end
	20	80,0	Yellow leaves and black cut end
	25	40,0	Yellow leaves and black cut end
AL	5	100,0	Green leaves / few black cut ends
	10	100,0	small shaded yellow/ few black cut ends
	15	20,0	Yellow leaves and black cut end
	20	30,0	Yellow leaves and black cut end
	25	20,0	Yellow leaves and black cut end

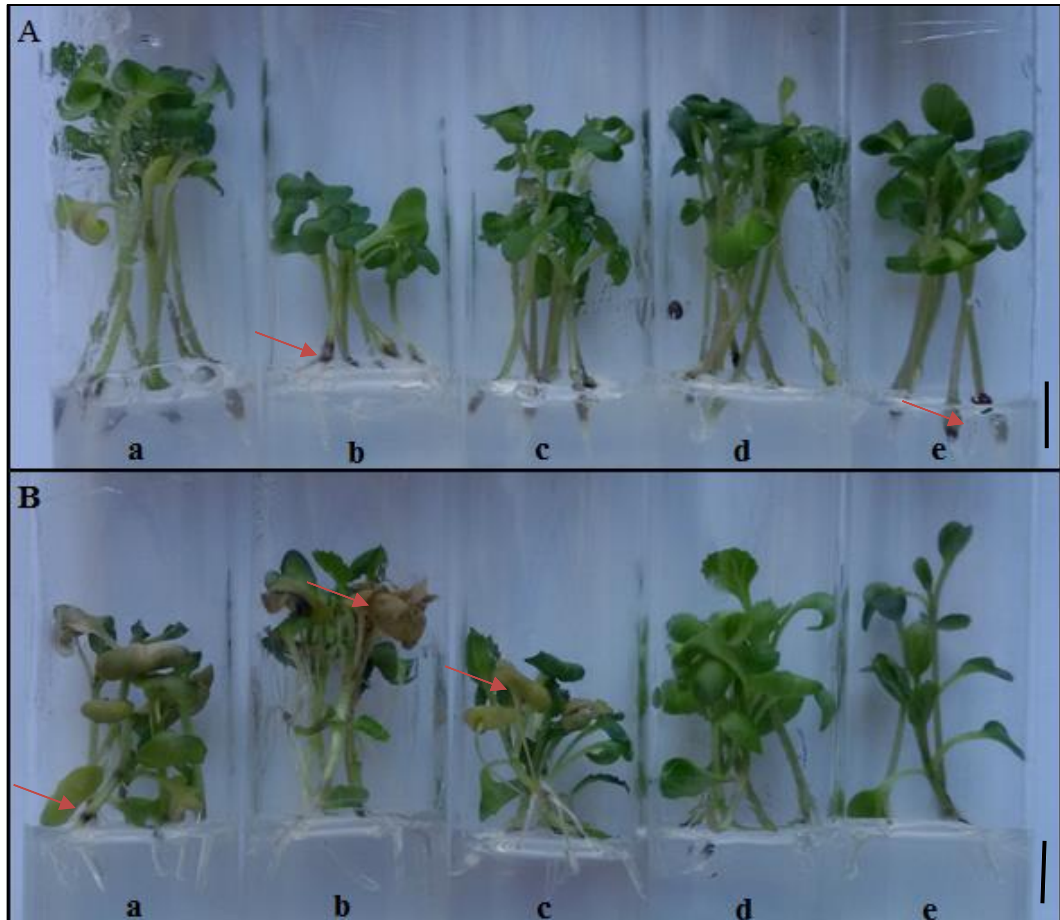


Figure 3.7: Analyzing optimum virulence concentration of *Fusarium* culture filtrate. 10 days old seedlings of cultivar PP (A) and Ki (B) in the MS shoot development medium (Appendix D) supplemented with series of concentrations of fungus culture filtrate (5%, 10%, 15%, 20% and 25% (v/v)) respectively “a” to “e” and Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine- HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8 after 30 days of incubation at 25 ºC ± 2 ºC in light. Arrows are pointed to the observed symptoms (black color cut ends of hypocotyls, yellow and dead cotyledons and leaves) of the disease. Bar scale = 1 cm.

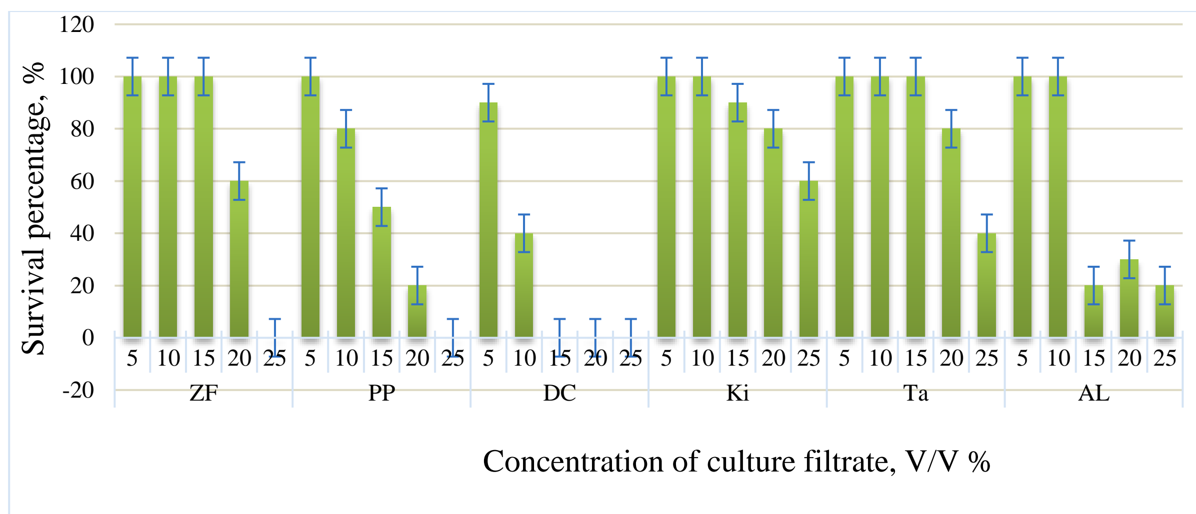


Figure 3.8: Survival percentage of seedlings of different cultivars under different phytotoxic pressures of *Fusarium oxysporium f.* Sp. *Conglutinans* culture filtrate.

3.3.2 Development of resistant plants through direct organogenesis

In the process of developing *in vitro* induced mutational resistance cabbage plants through direct organogenesis, explants excised from *in vitro* germinated cabbage seedlings were subjected to chemical mutagenic treatment prior to the culturing on MS shoot induction medium (Appendix B) and following regeneration (as described in chapter 2.2.2.3.3) of shoots from those treated explants, the selection of resistant plantlets were carried out.

3.3.2.1 Chemical mutagenesis and *in vitro* selection

Treated hypocotyls and shoot tip explants excised from 6 days and 10 days old seedlings, using chemical mutagens, dimethylsulphoxide (DMSO) (4% (v/v)) and ethyl methanesulfonate (EMS) (0.3% (v/v)) were cultured on MS shoot induction medium for several weeks.

Within the first week on the MS shoot induction medium following the mutagenesis treatment, the survival percentage of treated explants were significantly different between varieties, type of explant and age of the explant used (Figure 3.9 A1 and B1). In general, both 6 days old hypocotyl and shoot tip explants showed low survival percentage ranging from 8% to 46% and from 4% to 38% in hypocotyl and shoot tip explants respectively than 10 days old explants ranging from 8% to 60% and from 30% to 62% in hypocotyl and shot tip respectively. There was a significant difference of survival percentage between age and the type of explant. Six days old hypocotyl showed comparatively higher survival percentage than shoot tips while 10

days old shoot tips were having higher survival percentage than hypocotyls. But this survival percentage was significantly different between each cultivar as well. For example, in cultivar DC showed the lowest survival percentage, 8% of both age hypocotyl explant, but in the case of shoot tips, 6 days old shoot tips showed low survival (8%) and 10 days old shoot tips having higher survival percentage (36%). Both hypocotyls and shoot tip explants of cultivar Ta had the highest survival percentage (46% and 36% respectively) in six days old age group while hypocotyls of cultivar ZF (60%) and shoot tips of cultivar Ki (62%) were achieving that place in 10 days old age group.

During the first week on shoot induction medium, surviving explants were turned into green and during second week, hypocotyl explants become swollen and shoot tip explants showed growth of its main shoot increasing the size and length. Within 21 days, explants started to produce multiple shoots at the distal cut ends of the hypocotyl and base of the shoot tip (Figure 3.10) with different level of capacities among cultivars and age of the explants (Table 3.5).

There was not significant difference of shoot induction between types of explant but there was a significant difference between ages of the explants. As could observed (Figure 3.9), 10 days old explants showed higher percentage of shoot induction (lowest ranging from 8.0% to 30.0% and maximum ranging from 60.0% to 62.0%) than 6 days old explants (lowest ranging from 4.0% to 4.7% and maximum ranging from 40.0% to 38.0%). Among cultivars, there was significant difference of shoot induction between cultivars DC, Ki and Ta after mutagenesis treatment and there was not significant difference between cultivars PP, ZF and AL.

As in the direct organogenesis without mutagenesis treatment, the number of transferable shoots per explant were low compared to percentage of shoot induction. After mutagenesis, maximum number of shoots per explant was shown in 10 days old hypocotyl explants of cultivar PP (10.8 ± 0.3) and lowest was shown in 10 days old shoot tip explants of cultivar DC (0.0) (Table 3.5).
There was not significant difference of number of shoots induced per explant between types of explants but there was a significant difference between ages of the explants. Also between cultivars PP, ZF and DC, there was a significant difference of production of number of shoots per explant. Cultivar PP showed the maximum production of number of shoots per explant in both age groups and two types of explants (Figure 3.9. A3/B3 and Table 3.5). Most hypocotyl

explants of all cultivars were showed production of small callus at the cut edges of the hypocotyl after mutagenesis treatment.

Table 3.5: Percentage of explants developing shoots and shoots per explants produced from two different explants (excises from 6 days and 10 days old seedlings) of six cultivars after mutagenesis treatment through direct organogenesis.

		Explant			
		Hypocotyl		Shoot tip	
Cultivar		Explants giving shoots (%)	Average no. of shoots/explant	Explants with shoots (%)	Average no. of shoots/explant
ZF	6 Days	30,0	1,6±0,1	32,0	2,8±0,4
PP		40,0	4,1±0,2	28,0	6,4±1,2
DC		4,7	0,6±0,4	8,0	0,7±0,5
Ki		10,0	2,2±1,5	4,0	3,2±2,3
Ta		30,0	1,3±0,7	38,0	1,5±1,1
AL		10,0	0,6±0,5	10,0	1,8±1,3
ZF	10 Days	60,0	4,0±0,1	56,0	0,2±0,1
PP		50,0	10,8±0,3	46,0	7,2±0,7
DC		8,0	1,0±0,8	36,0	0,0
Ki		16,0	1,3±0,1	62,0	0,5±0,1
Ta		40,0	1,1±0,1	46,0	0,8±0,1
AL		34,0	1,1±0,1	30,0	0,8±0,3

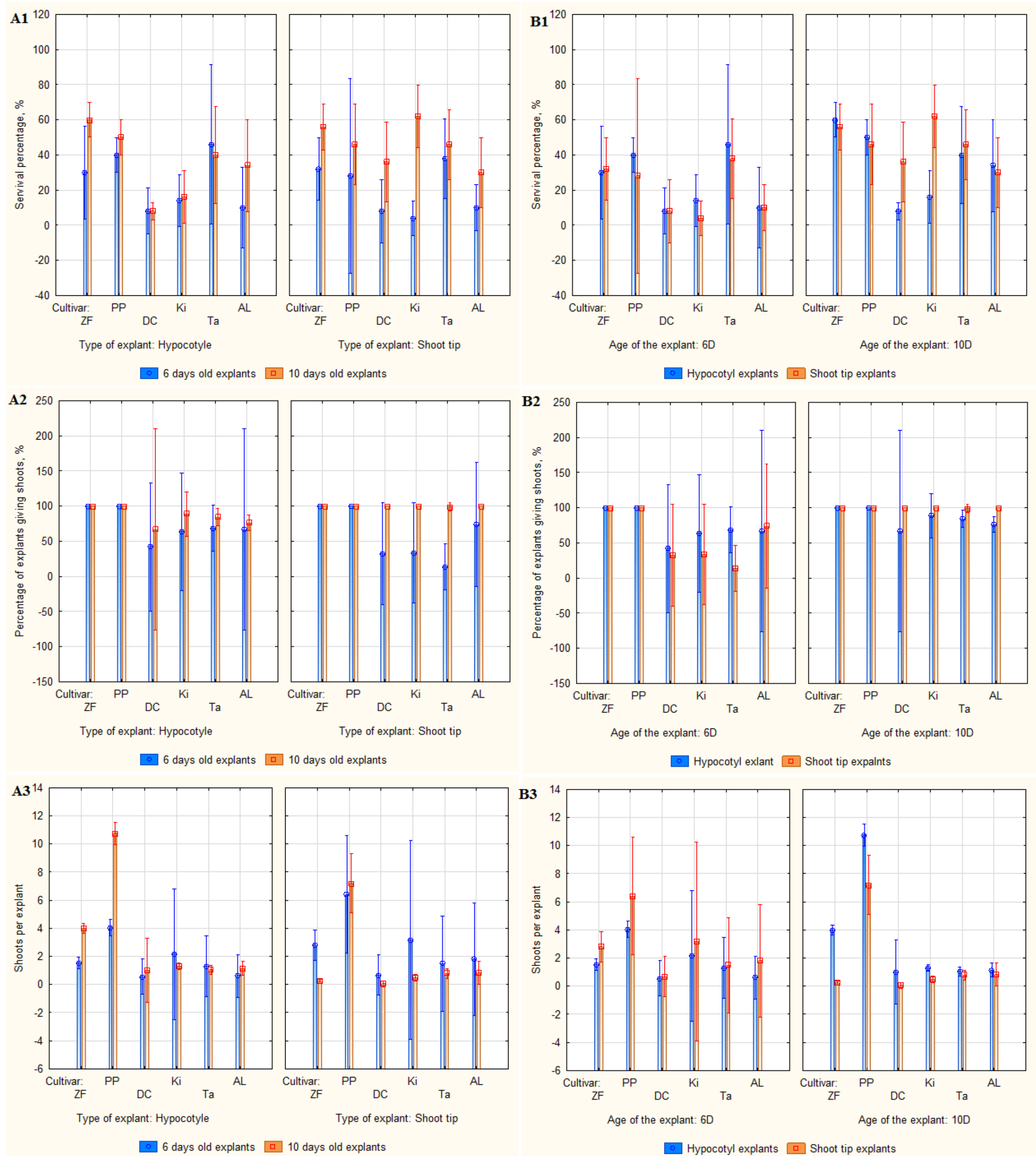


Figure 3.9: Different responses of different explants of two different age groups of six different cultivars after mutagenesis treatment.

A. Comparison of survival percentage, percentage number of explants inducing shoots and number of shoots per explants, between two age groups of different explants in each cultivar.

B. Comparison of survival percentage, percentage number of explants inducing shoots and number of shoots per explants, between two different explants of two different age groups in each cultivar.

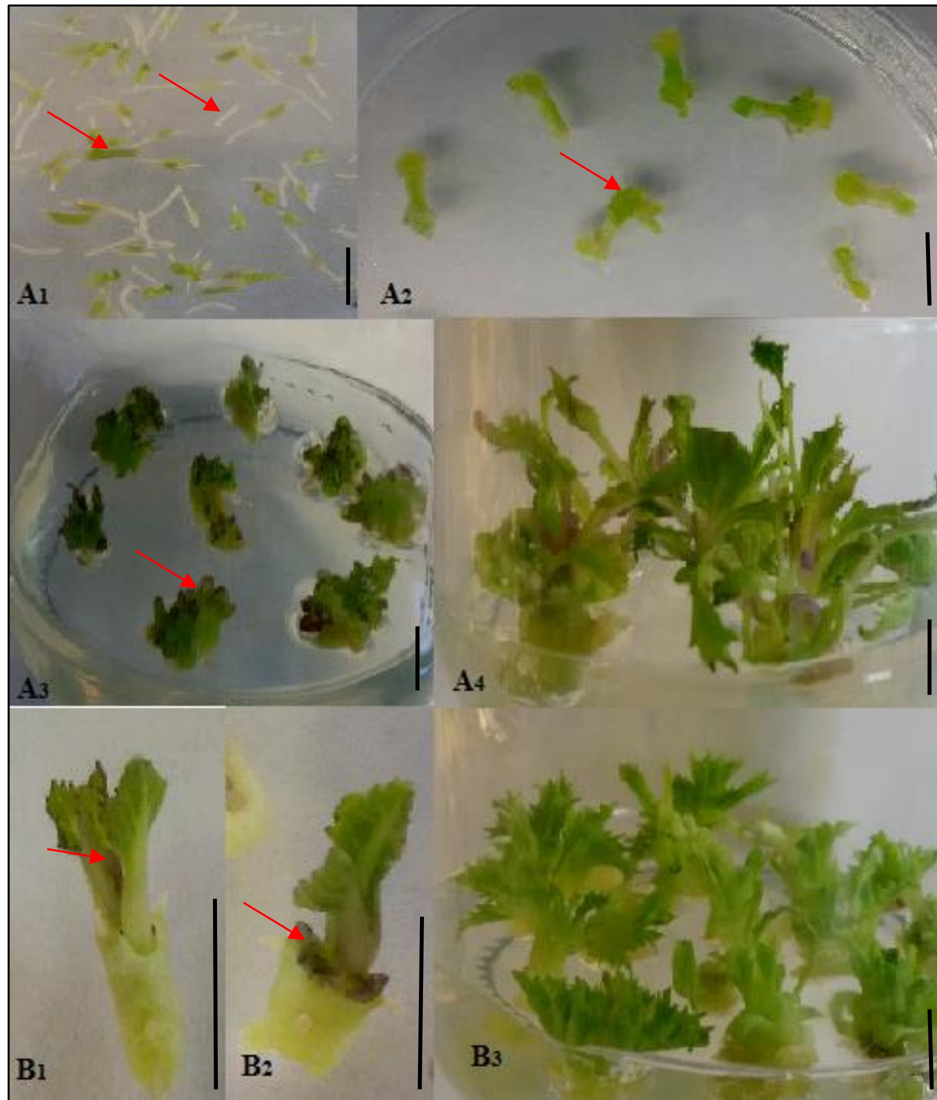


Figure 3.10: Direct shoot induction from hypocotyl and shoot tip explants excised from 6 days old seedlings, in MS shoot induction medium. (15 mL) (Appendix B) supplemented with Nicotinic acid (1 mg/L), Thiamin-HCl (10 mg/L), Pyridoxine-HCl (1 mg/L), Myoinositol (100 mg/L), NAA (0.2 mg/L), BAP (3 mg/L), GA_3 (0.01 mg/L), $AgNO_3$ (0.5 mg/L), Sucrose (20 g/L), and phytagel (7 g/L) in pH 5.8. Cultures were incubated for three weeks at 25 ºC ± 2 ºC in light. Arrows are pointed to the inducing and developing shoots. Bar = 10 mm

A. Hypocotyl explant; A1. Green color surviving explants and dead explants after 7 days in MS shoot induction medium, A2. Swelling and callus like structure formation after 14 days, A3. Clear multiple shoot induction after 30 days- A4. Well grown shoots after 45 days.
B. Shoot tip explants; B1. Growth of main shoot and increased size of explant after 7 days, B2. Multiple shoot induction at the base of the shoot tip after 14 days, B3. Clear multiple shoot development after 21 - 30 days.

Selection of shoots carrying resistance to *Fusarium* culture filtrate;
After successful shoot induction and development through direct organogenesis following the mutagenesis, Shoots were subjected to selection pressure under 15% v/v concentration of *Fusarium* culture filtrate for 30 days and another 60 days under 20% v/v concentration for further selection to be stabilize the resistance in surviving shoots. During the first selection cycle under 15% selection pressure most shoots were died isolating the shoots which can tolerate or resist to phytotoxic effect of culture filtrate. In some cultivars, it was over 50%. During the second cycle of selection under higher selection strength, the mortality rate was much lower compared to first selection cycle.

Table 3.6 shows the data of in both selection cycles and root induction capacities of selected shoots. The percentage of the number of survival shoots after both selection cycles was not significantly difference within either factor; type of explant or age of the explant, but there was a significant different between some cultivars and between cultivar- type of explant- age of the explant (Figure 3.11). Even though, there was no significant difference, observed data were showed some differences between each factor. cultivars; PP, ZF, Ki, AL and Ta showed increased resistance development rate using six days old shoot tip explant (ranging from 36.7-86.7%) than hypocotyl explant (ranging from 16.7 – 28.3%) while cultivar; DC was showing reduction of resistant mutation development in case of both age groups (from 66.7% to 16.7% in 6 days old explant and from 46.7% to 0.0% in 10 days old group). Except cultivars ZF and AL, all other cultivars showed reduction of resistance mutation induction from 10 days old hypocotyl explant to shoot tip explants. There was also a slight increase of resistance development with the increase of the age of hypocotyl explant and shoot tip showed small reduction from 6 days to 10 days old.

There was a significant difference of shoot induction from each type of explant among cultivars without subjecting to mutagenesis treatment with selection and after mutagenesis and selection. But there was not significant difference of shoot induction between without and with mutagenesis treatment and selection, among age of the explant and among hypocotyl and shoot tip explant (Figure 3.13A1/A2).

Though statistically there was not significant difference of shoot induction without and after mutagenesis treatment, observable data showed reduction of shoot induction from 6 days old hypocotyl after the mutagenesis treatment with compared shoot induction without mutagenesis treatment in all cultivars except Ki. Ki showed opposite response. Significant increase of production of number of shoots per explant from 10 days old hypocotyl of cultivar PP was observed after the mutagenesis treatment. In case of shoot tip explant, except cultivar ZF and DC, 6 days old shoot tips of other cultivars showed increase of shoot production after the mutagenesis treatment and from 10 days old shoot tips, only cultivar PP was showed increase of shoot induction after the mutagenesis treatment (Figure3.13A1/A2).

3.3.3 Development of resistant plants through indirect organogenesis

Shoots which supposed carrying resistance to *Fusarium* were achieved from the calli (which were derived from 6 days old hypocotyl explants) which were selected using Fusarium culture filtrate following the chemical mutagenic treatment.

3.3.3.1Chemical mutagenesis and *in vitro* selection

Following the successful mutagenic treatment with dimethylsulphoxide (DMSO) (4% (v/v)) and ethyl methanesulfonate (EMS) (0.3% (v/v)), calli were incubated on callus induction medium (Appendix C) containing fungus culture filtrate (15% (v/v)) for 30 days at 25 ºC ± 2 ºC in light.

After 30 days, surviving calli were incubated for another 60 days on same callus induction medium containing (20% (v/v)) at 25 ºC ± 2 ºC in light. Surviving calli were transferred into same fresh medium with 20% v/v fungus culture filtrate after 30 days.

Then shoots were developed form selected surviving calli through shoot induction and development as same as described above in chapter 2.2.1.3.4.2 and 2.2.1.3.5. Figure 3.12 Shows the different stages of selection and shoot induction during the process of developing resistance plantlets through indirect organogenesis pathway.

After mutagenesis treatment and *in vitro* selection, surviving yellow-green calli were started shoot induction in the first week on shoot induction medium. Some calli of some cultivars were showed shoot induction even in the second cycle of resistance selection under 20% selection pressure. But most of remained calli after selection were showed dormant behavior in the shoot induction medium and they were green and hard compact calli without any shoot induction even after 60days in the shoot induction medium. This dormancy was greatly observed in cultivar DC with zero shoots per calli. Generally, the average number of shoots per calli were very low (Table 3.8 and Figure 3.13A) in all cultivars after mutagenesis and selection. Maximum average number of shoots per calli were observed in cultivar Ki (0.8±0.1) and Cultivars ZF and PP showed similar average number of shoot induction. But there was not any significant difference of survival percentage of calli after mutagenesis and selection among different cultivars but there was a significant difference of shoot induction among cultivars.

Interestingly as seen in direct organogenesis as well, there was a significunt difference of indused number of shoots per calli between shoot induction without subjecting to mutagenesis treatment with selection and after mutagenesis and selection (Figure 3.13B) and shoot induction was reduced after mutagenesis.

Table 3.6: Selection of shoots developed from two types of explants with two different age after mutagenesis and root induction of selected *Fusarium* resistant shoots.

Age of the initial explant	Type of explant	Cultivar	No. of shoots	Percentage of survival under 15% selection strength, % (After 30d)	Percentage of survival shoots under 20% selection strength, % (After 60d)	Total no. of rooted shoots (after 30-45days)	percentage of root induction %
6D	Hypocotyl	ZF	60	36,7	16,7	9	90,0
		PP	60	46,7	21,7	11	84,6
		DC	6	66,7	66,7	0	0,0
		Ki	45	26,7	26,7	8	66,7
		Ta	60	35,0	28,3	4	23,5
		AL	15	13,3	13,3	1	50,0
	Shoot tip	ZF	60	75,0	65,0	27	69,2
		PP	60	56,7	50,0	24	80,0
		DC	6	50,0	16,7	1	100,0
		Ki	15	93,3	86,7	9	69,2
		Ta	18	44,4	44,4	6	75,0
		AL	30	60,0	36,7	4	36,4
10D	Hypocotyl	ZF	60	28,3	16,7	9	90,0
		PP	60	81,7	81,7	45	91,8
		DC	15	46,7	46,7	2	28,6
		Ki	25	88,0	84,0	11	52,4
		Ta	50	36,0	36,0	12	66,7
		AL	40	12,5	12,5	4	80,0
	Shoot tip	ZF	20	60,0	60,0	12	100,0
		PP	60	5,0	35,0	18	85,7
		DC	0	0,0	0,0	0	0,0
		Ki	30	60,0	60,0	10	55,6
		Ta	50	42,0	26,0	9	69,2
		AL	35	37,1	31,4	7	63,6

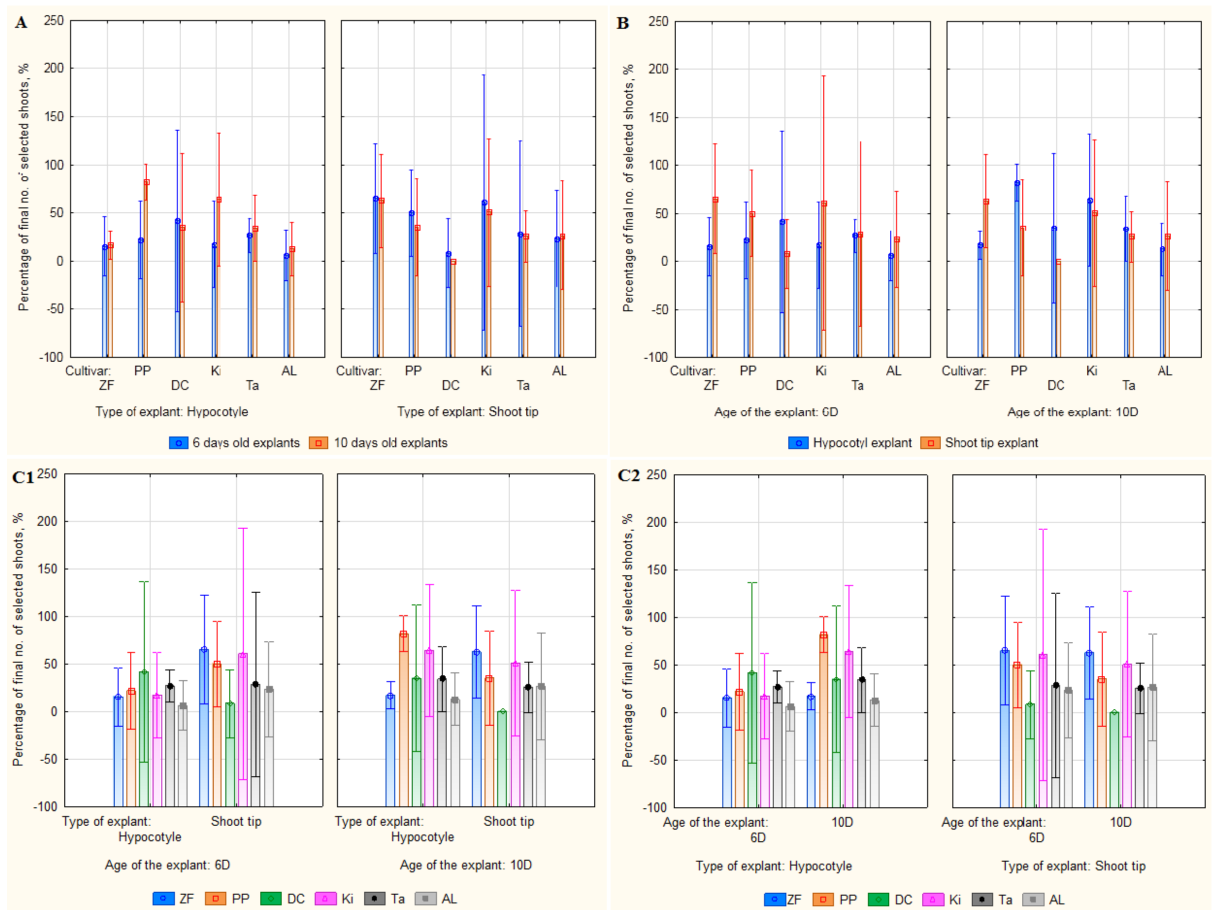


Figure 3.11: Selection of shoots having *Fusarium* resistance, developed through direct organogenesis after mutagenesis treatment.

A. Comparison between two age groups of different explants developing *Fusarium* resistant shoots.

B. Comparison between different explants with different age groups developing *Fusarium* resistant shoots.

C. Comparison between behaviors of cultivars developing resistance shoot from two different explants under two age groups.

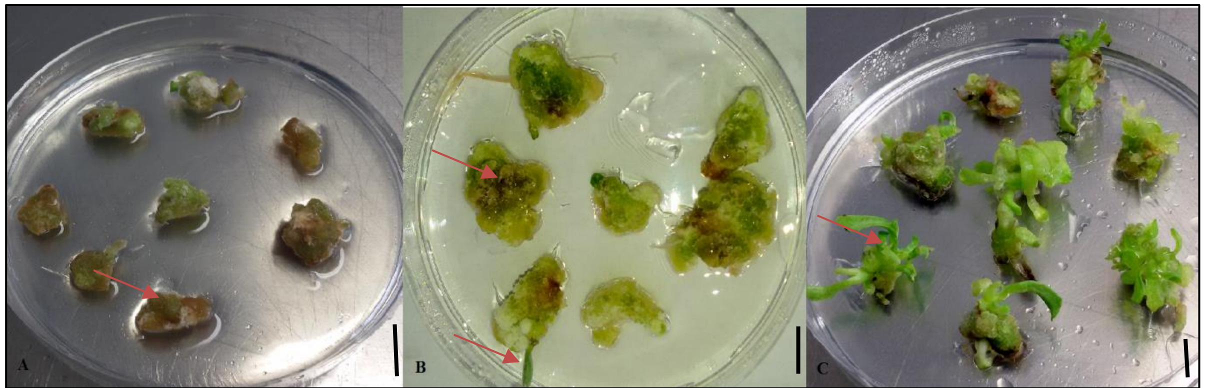


Figure 3.12: Selection of calli and induction of shoots carrying *Fusarium* resistance after mutagenesis treatment. Bar = 10 mm.

A. Treated calli on the selection medium consisting *Fusarium* culture filtrate (15% v/v) after 14 days. Arrows are pointing the greenish alive calli cells which shows resistance and dead calli cells which are sensitive to phytotoxics in the culture filtrate.
B. Selected calli by 20% v/v culture filtrate showing averagely zero percentage shoot induction on shoot induction medium after 30 days. Arrows are pointing to the dormant callous and small rear shoot induction on one callus.
C. Inducing multiple shoot from selected calli on shoot induction medium after 21 days of incubation at 25 ºC ± 2 ºC in light.

Table 3.7: selection of treated calli and shoot induction after selection of mutated calli carrying resistance to culture filtrate.

Cultivar	Mean survival calli on 20% selection strength	Average no. of shoots achieved	Ava. Shoot/calli
ZF	46,0±9,4	13,0±2,9	0,6±0,1
PP	42,7±15,5	12,0±2,4	0,6±0,1
DC	30,0±19,6	0,3±0,5	0,0
Ki	46,0±19,8	18,0±2,9	0,8±0,1
Ta	36,7±13,6	5,0±2,2	0,3±0,1
AL	48,7±5,1	2,0±1,6	0,1±0,1

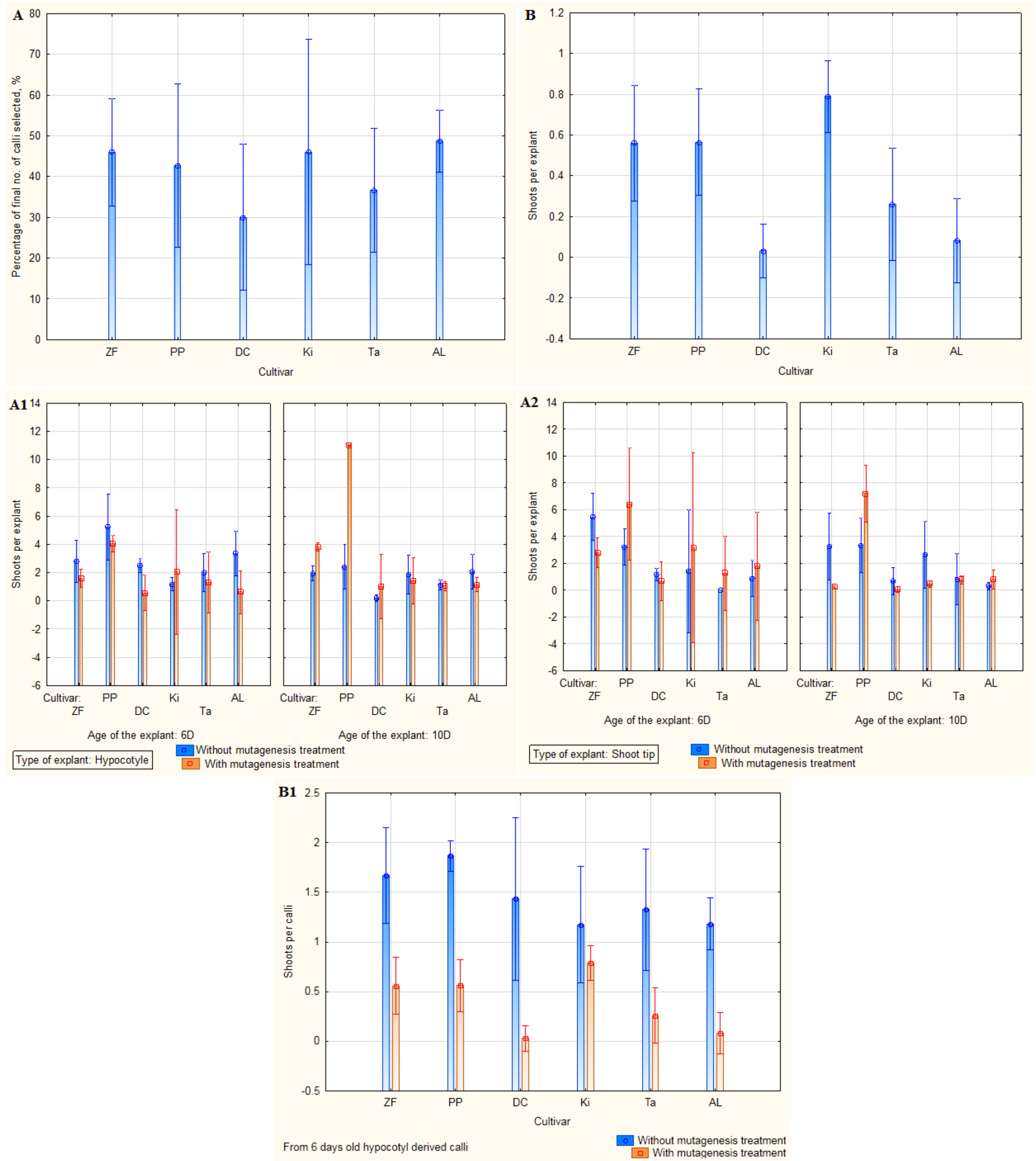


Figure 3.13: Development of resistance shoots from indirect organogenesis after mutagenesis and comparison between shoot induction without and with mutagenesis treatment.

A. Differences between developments of calli carrying Fusarium resistance calli from 6 days old hypocotyl derived calli after mutagenesis treatment in each cultivar.
B. Differences between shoots per calli developed by selected calli (derived from 6 days old hypocotyl) carrying Fusarium resistance after mutagenesis treatment in each cultivar.

A1. A2. And B1. Comparison between shoot induction through both direct and indirect organogenesis, before mutagenesis treatment and after mutagenesis treatment.

3.3.4 Root induction and acclimatization of *in vitro* developed resistant cabbage plants

Figure 3.14: Root development and acclimatization of selected *Brassica* plantlets which supposed to carry *Fusarium* resistance. Bar = 1cm

A. Root development in MS root induction medium (Appendix E) supplemented with IBA (0.2 mg/L), after 30 days.
B. Fully developed plantlets which are ready to transfer to soil.
C. Acclimatization of *in vitro* developed Fusarium resistant plantlets, after 21 days.
D. Fully acclimatized plantlet into normal environment.

As seen in the shoots developed *in vitro* without subjecting to mutagenesis treatment, shoots developed through mutagenesis and selection steps, root induction was appeared within 21 days with some exceptions in some cultivars; for example, DC (0-2 shoots with root induction after 30days). Cultivars ZF and PP were showed better root induction compared to other cultivars. They produced dense and long (>3.0 cm) roots (Figure 3.14) within 21days in root induction medium (Appendix E) supplemented with IBA (0.2 mg/L). But these observed different data of root induction capacities between different cultivars is not much precise due to very low number of shoots achieved after mutagenesis and selection process (Table 3.7).

During the Acclimatization process, higher mortality rate was observed among all cultivars (50 – 100%). Successfully acclimatized plants shows normal phenotypic appearance (Figure 3.14).

CHAPTER 04

Discussion and conclusion

Chapter 04

Discussion and Conclusion

4.1. Discussion

As a widely growing *Brassica* vegetable worldwide and as an important crop which carries lot of nutritional and some medicinal properties, improvement of quality performances of *B. oleracea* var. *capitate* (white cabbage) and develop new genotypes is important. *Fusarium* wilt is a concerning fungal disease which affect to the quality and the production of cabbage. As the only applicable controlling method is cultivation of *Fusarium* resistance cultivars, lack of appropriate *Fusarium* resistant traits within the variety is a big problem in breeding programs developing new cabbage varieties carrying Fusarium resistance. Mutational breeding is a good approach in this situation. *In vitro* mutation induction is an important technique that can be used to achieve new resistance lines for future breeding processes.

In the *in vitro* tissue culture process, having a reliable and efficient culture protocol is a critical point of the success. In this case, selection of appropriate explant and media compositions are key points. There are various types of explants that can be used; hypocotyls, leaf, cotyledons, shoot meristems, nodes, pollen etc. There are many published studies in *Brassica* species using different explants. In this study, explants were selected from *in vitro* germinated seedlings. Use of explants from *in vitro* germinated seedlings has many advantages including reduction of risk of contamination during tissue culture process and avoid the effect on regeneration capacity due to stress of chemicals used for surface sterilization (Yildiz, 2002) when using explants from outside grown plants. It also provides large number of explants under aseptic conditions over a short period year-round. Moreover, it has reported a relatively higher shoot organogenesis from *in vitro* derived explants (Burbulis et al., 2008). Different studies related to different varieties of *Brassica oleracea,,* different conditions have been used for in *vitro* seed germination such as use of wet sterilized papers in aseptic conditions (Qamar et al., 2014), half-strength or full strength MS medium supplemented with different amount of sucrose (30 g/L, 20 g/L, 10 g/L) with or without plant growth regulators and different amount of either agar (8 g/L, 2.5 g/L) or phytagel (7 g/L) (Gerszberg et al., 2015; Handyani, 2014; Pavlović et al., 2012; Bhalla and Weed, 1999; Bhalla and Smith, 1998). There are not clear data mentioned about the success of each of those germination conditions. In this study, growth regulator free MS medium supplemented with sucrose (10g/ L) and phytagel (7g/L) in pH 5.8 was used which was successfully used by Bhalla and Smith (1998) for *in vitro* germination of cauliflower (*B.*

oleracea var. *botrytis*) for the excision of explants for *Agrobacterium* mediated transformation. Gerszberg et al. (2015) also used same growth regulator free MS medium with a 0.8 agar instead of phytagel as a gelling agent for invitro germination of some cultivars of *B. oleracea* var. *capitata* and they achieved maximum 90.7 % germination of some of tested cultivars after 10 days. Phytagel refers as less free mineral containing and with less impurities than agar (Pasqualetto *et al*., 1986 and 1988). Per William *et al*., 1990, the pH of the medium with phytagel stay stable longer than media with agar. In this study, only the cultivar Ki showed the lowest germination percentage (44.0%) and ZF showed 94.0% while all other cultivars achieving 100% germination after 7 days and 2.5 cm – 3.5 cm height.

Combination of proper plant growth regulators in correct concentrations determine the efficiency and the capacity of the organogenesis in in vitro culture process. Previous studies have been shown that presence of BAP either alone or in combination with auxin (mostly NAA) optimize the shoot regeneration and multiplication in different Brassica species (Metz et al., 1995; Bhalla and Weed, 1999; Jin et al., 2000; Cheng et al., 2001; Munshi et al., 2007; Sretenovič – Rajičić et al., 2007; Maheshwari et al., 2011; Pavlović et al., 2012). Cheng et al. (2001) showed that the presence of BAP, significantly increase the number of shoots per explant and NAA significantly enhance the shoot regeneration (Guo et al.,2005) in B. oleracea in vitro cultures. Some of previous studies have also showed the combination of higher concentration of auxins and lower concentration of cytokinin are more efficient for callus induction (Maeshwari et al., 2011; Qamar et al., 2014; Liu et al., 2015). For this study, NAA (0.2 mg/L), BAP (3 mg/L) and GA3 (0.01 mg/L) were used for direct shoot induction and same amount of NAA and BAP were used excluding GA3 for callus induction. Even though different study groups have tested and used different concentrations of these plant growth regulators for in vitro propagation and transgenic development of different varieties of B. oleracea, most of studies noticed an efficient shoot induction and development especially from the hypocotyl explants in the media containing BAP (3mg/L) and NAA (0.2 mg/L) (Bhalla and Weerd, 1999; Mollica et al., 2011; Handayani, 2014; Gerszberg et al., 2015). Incorporation of GA3 into the shoot induction and development medium can have either beneficial or negative effect depending on the plant species. Some research studies observed multiple shoot induction (in tomato; Bima et al., 1994), shoot and root induction (Buckwheat; Srejović and Nešković, 1985), elongation of induced shoots (Sweet potato; Ndagijimana et al., 2014). Bhalla and Smith (1998) and Bhalla and Weerd (1999) used combination of BAP, NAA and GA3 for regeneration of shoots from B. oleracea var. botrytis and achieved 88% shoot indu

ction and 6.1 ± 3.0 of average number of shoots per stem explant (Bhalla nad Weerd, 1999) and 85- 99% of shoot induction from hypocotyl explants (Bhalla and Smith, 1998). In this study, with mentioned growth regulator combination, most cultivars were showed more than 50% shoot induction from both hypocotyl and shoot tip explants with 5.9±0.6 of maximum number of shoots per explant (from shoot tip) (Table 3.1).

Though there are various explants that can be used as a starting material of plant tissue culture, all these tissues are not suited for all *in vitro* organogenesis procedures and for all plants. Different explants of plant species have different efficiencies in *in vitro* cultures. The regeneration efficiency very dramatically depends on the explant type of the genotype (Mollika et al., 2011), age of the explant (Ovesna et al., 1993) due to the physiological status (Liu et al., 2015). Three types of explants; hypocotyl, shoot tip and cotyledon with two age groups; 6 days and 10 days from seedlings were tested for regeneration capacity of selected cultivars of *B. oleracea* var. *capitata* in this study. As previous studies, have been reported, significant difference of regeneration between different explants, age and cultivar was observed in both direct and indirect organogenesis path ways. In direct and indirect organogenesis analysis, cotyledon explant was showed lowest shoot production (ranging from 0 to 30% shoot induction and 0 to 2.0 ± 1.6 shoots per explant in direct organogenesis and 0 to 90% of callus induction with 0 to 1.6 ± 0.8 shoots per callus in indirect organogenesis) in both age group. Gerszberg et al. (2015) also observed the similar very low percentage of organogenesis from cotyledon explants of some cultivars of *B. oleracea* var. *capitata*. But, Qamar et al. (2014) observed 100% callus induction from hypocotyl of cabbage cultured on the medium supplemented with 2,4-D (2.0 mg/L) and BAP (0.25 – 1.0 mg/L) but considerably higher amount of cytokinin was essential for shoot induction form that callus to break the strong auxin effect of 2,4-D. They achieved 100% of shoot induction by combination of BAP (4mg/L), Zeatin (4mg/L) and IAA (5mg/L). Munshi et al. (2007) also observed maximum percentage of callus induction from cotyledon explants on 2,4-D (1.0 mg/L) and NAA (0.5 mg/L) and shoot induction was only observed from the calli derived from cotyledon explants.

In the direct organogenesis of this study, 10 days old hypocotyl explants showed maximum shoot production (in general 100%) while 6 days old hypocotyl resulting higher number of transferable shoots per hypocotyl (5.2 ± 0.8) compared to 10 days old hypocotyl. Shoot tip explants also showed the similar response with maximum shoot induction in 10 days old shoot tip (maximum 92%) and better production of shoots per explant in 6 days old explants

(maximum 5.9 ± 0.6). Each cultivar was showed different regeneration capacities in each type of explants. Cultivars PP and ZF showed better regeneration response in all three explants and ages compare to other four cultivars tested (Graph 3.2). As could observed and analyzed data, 6 days old hypocotyl explants showed significant better responses for the used tissue culture protocols with some exceptions between cultivars. In the indirect organogenesis, also hypocotyl explants were showed better response on callus induction (46.0 – 95.3% callus induction) with a significant difference in shoots per explant between different cultivars.

Ovesna et al., (1993) has reported that young explants give better shoot induction than older explants and most studies have shown that explants excised from 4-5 days old seedlings result optimal regeneration rate in different *Brassica* spp. (Sharma et al., 1990; Hachey et al., 1991; Bhalla and Smith, 1998; Mollika et al., 2011). But due to difficulties with the very small size of explants from such very young seedlings, in many studies, used explants excised from 5-7 and even from 10 days old seedlings (Munshi et al., 2007; Pavlović et al., 2010; Rafat et al., 2010; Handyani, 2104; Gerszberg et al., 2015). Also, previous studies have reported, hypocotyl as a suitable explant for *Brassica in vitro* regeneration (Tang et al., 2003; Li et al., 2005; Zhang et al., 2006; Sretenović – Rajičić et al., 2006; Munshi et al., 2007; Khan et al., 2010; Rafat et al., 2010). In this study, as well, 6 days old hypocotyl explants showed the better response towards *in vitro* organogenesis through direct and indirect path ways for most of cultivars tested.

Low survival percentage (< 50% in 6 days old explants and <62% in 10 days old explants) of explants after chemical mutagenesis treatment was observed during this study. In this study explants were exposed to mixture of DMSO (4% (v/v)) and EMS (0.3% (v/v)) for two hours. The dosage of the mutagen treatment is important to achieve desirable mutation effect due to random and unknown degree of mutations that can occur within the target genome. Though higher dosage increase the degree of mutation rate it will result high lethality of plant materials. The LD50 value of mutagen can be varies depending on the plant species, type of plant material etc. For example, for *Gossypium hirsutum,* 3% v/v EMS (Auld et al., 1998), for banana, 4% v/v DMSO and 0.3% v/v EMS (Matsumoto et al., 1999b), Microspore of *B. napus;* 0.25% v/v EMS (Ali and Ahmad 2004), for sugar beet seeds; 0.5% v/v EMS (Hohmann et al., 2005), for Saintpaulia; 0.4% v/v EMS (Yi Fang, 2011). Therefore, detection of effective and efficient mutagen does-response relationship is important. Nova (1991) have reported that the efficiency of chemical mutagenesis is depended on chemical and physical properties of the mutagenic

chemical, solubility of the mutagen, temperature, light, pH of the solution, oxygen availability during the treatment, capacity of absorption, method of application, size of the plant materials, post-treatment washing etc. Van Harten (1998) also emphasized the low penetration of mutagenic chemical into plant tissues lead to low mutation efficiency and difficulties in reproducing the experiment (Van Harten, 1998). In addition to higher mortality of plant materials with the increase of the strength of the mutagen, it also reduces the regeneration capacity of the plant materials. Yun et al., (2005) has observed, zero percent regeneration from cotyledon explants of *B. rape* after EMS (1% v/v) treatment for 25 hours and after treating with NaN_3 (1000µM) for 25hours. Ali and Ahmad (2004) also observed 25- 40% reduction in regeneration from microspore of *B. napus* even after treating with lower concentration of EMS (0.25%) compared to untreated microspore. During current study, was also observed reduction of regeneration capacity (maximum average number of shoots per calli; 0.8 ± 0.1 after mutagenesis while being 1.7 ±0.2 of maximum without mutagenesis treatment) through indirect organogenesis (from calli derived from 6 days old hypocotyl) after treatment showing dormancy in most of calli of all cultivars (Figure 3.13) with maximum shoot production from cultivar Ki compared to all 6 cultivars. But through direct organogenesis, observed results were varies and interesting among cultivar, explants and age of the explants. As previous studies, have been noticed, here also reduced shoot production was observed especially from 6 days old hypocotyl explant in each cultivar except cultivar Ki which showed small increase of shoot induction (but not significant) after mutagenesis treatment. Interestingly, increased shoot production was observed from explants of cultivar PP (10-day old hypocotyl, and both 6 and 10 days old shoot tip explants) after the treatment. In generalize view, 6 days old shoot tip explants were showed increased shoot induction in each cultivar except cultivar ZF and DC (Figure 3.13). This is an interesting observation due to none of previous reported studies on *Brassica in vitro* mutagenesis have studied about use of shoot tip explant.

Selection of efficient selection agent is important to achieve efficient success in screening mutant resistance in *in vitro* conditions. Cells free culture filtrate of *Fusarium oxysporium* has been successfully applied for *in vitro* selection of many species so far; turmeric (Matsumoto et al., 1999: *Musa spp;* Krause et al., 2003: *Linum*; Kuanar et al., 2014: turmeric: Kantoglu et al., 2010: melon). Karaman and Matayuly (2005) reported that variety of mycotoxins and enzymes present in the culture filtrate of *F. oxysporum* culture filtrate may be attributed to reduction of growth and alteration in physiological parameters which are related to virulence of the fungus.

In this study, as well *Fusarium* culture filtrate was successfully applied achieving efficient selection responses.

The detection of selectable toxicity of selection agent is also another critical point in *in vitro* mutagenesis and selection of desired mutants. In this study, cells free *Fusarium* culture filtrate was used as the selection agent. The effective concentration for selection process were determined by a preliminary experiment using *in vitro* germinated 10 days old seedlings and series of concentrations of culture filtrate. The analysis was done based only on the morphological changes as like symptoms of *Fusarium* infection (necrosis, chlorosis, discoloration of vascular tissues etc) and mortality rate of the seedlings. With the increase of concentration of culture filtrate, mortality rate was increased in all cultivars reaching to 100% mortality of most cultivars (ZF, PP, DC) at the 25% v/v concentration. Cultivar DC showed higher sensitivity showing mortality response even in low concentration and 15% v/v was 100% lethal for DC while cultivar Ki were showing low sensitivity even to highest concentration tested (25% v/v, with 60% survival percentage). But after mutagenesis treatment, various percentage of resistance towards *Fusarium* culture filtrate was observed depending on type of explant and age among cultivars (Table 3.7 and Figure 3.11). Maximum resistance was observed in shoots developed from 6 days old shoot tip explants of cultivar Ki (86.7%) under 20% selection strength. It was an increase of resistance to *Fusarium* culture filtrate compared to resistance showed during lethality test (80%) and cultivar DC which showed higher sensitivity (100% mortality under 20% section strength) was showed increase resistance after mutagenesis accomplishing maximum resistance (66.7%) in shoots derived from 6 days old hypocotyl revealing induction of resistance to *Fusarium* by mutagenesis treatment. Depending on the results observed in a generalize view, 10 days old hypocotyl and 6 days old shoot tips are suitable for development of mutational resistance to *Fusarium* in *B. oleracea* var. *capitata.*

During this study, vitrification (hyperhydricity) of shoots were observed. Presence of excess amount of cytokinin along with the high-water potential in the medium would be the reason for this vitrification (Li et al. 2003). Pavlović et al. (2010) observed a high percentage of vitrification in shoots of cauliflower and savoy cabbage cultures and in *B. oleracea* var. *capitate* (9.09% to 81.77%) (Pavlović et al., 2012) which was containing BA. Vandemoortele et al. (2001) has reported a simple method for propagation of cauliflower without vitrification using osmotic pretreatment by soaking explants in sucrose solution (2 MPa for 24 hours) before culturing on the plant growth regulator free medium. Pavlović et al. (2010) have substituted

BA with KIN to overcome this vitrification lowering (>50%) in cauliflower achieving satisfactory regeneration and around 50% in cabbage but couldn't achieve satisfactory regeneration of shoots (Pavlović et al., 2012) using KIN.

Another problem observed during this study was chlorosis and necrosis of cotyledon explants leading to death of cotyledons with the time of culture. Many other study groups observed the similar effect on *Brassica* tissue culture (Qin et al., 2007; Rho et al., 2012; Gerszberg et al., 2015) and there are several factors that can be reasons for this problem. Accumulation of phenolic compounds and their oxidation products could be a one reason for this. *Brassica* species are rich sources of phenolic compounds. Accumulation of ethylene produced from *in vitro* growing plant tissues due to wounding stress can be another reason for this chlorosis and necrosis. Accumulation of ethylene can also cause poor regeneration capacity or abnormal growth of shoots (chi and Pua 1989; Chi et al., 1991; Eapen and George., 1997) by inhibiting cell division, DNA replication, growth in meristems of roots, shoots and auxiliary buds (Gane., 1934; Apelbaum et al., 1972). Use of ethylene inhibitors is important in *Brassica in vitro* regeneration and it is refered as a necessity in *in vitro Brassica* regeneration (Hee et al., 2012). Many study groups observed the enhanced shoot regeneration in *in vitro* cultures of *Brassica* species with the use of ethylene inhibitors (Chi and Pau., 1989; Pental et al., 1990; Pua and Chi. 1993). Silver nitrate ($AgNO_3$) is widely used ethylene inhibitor in many previous studies of *Brassica* micropropergation. $AgNO_3$ not only act as an ethylene inhibitor but also enhance the regeneration efficiency of *in vitro Brassica* cultures (chi et al., 1990; Zhang et al., 1998; Qin et al., 2007; Cogbill et al., 2010; Maheshwar et al., 2011). But, Radlke et al. (1992) reported that the continuous exposure to $AgNO_3$ can lead to vitrification of shoots. In this study $AgNO_3$ was incorporated only into shoot induction medium and eliminated from other media used. Bhalla and Weerd (1999) also was used $AgNO_3$ only for the shoot induction from explants of *B. oleracea* var. *botrytis*. Bhalla and Weerd (1999), Qin et al. (2007) and Gerszbery et al. (2015) have proposed that low air exchange of *in vitro* cultures might be a reason for ethylene accumulation. Bhalla and Weerd (1999) has suggested that use of porous surgical tape (3M micropore) as sealing material of culture plats rather than use of non-porous plastic (Nescofilm or parafilm) to increase the air exchange and reduce the accumulation of ethylene and they also observed the reduction of condensation in the cultures which was another problem observed in the present study as well. John et al. (1991) reported the reduction of necrosis in cotyledon explants of *B. compestris* by lowering the light intensity from 60 – 80 $\mu Em^{-2}S^{-1}$ to 30 – 40

μEm^{-2}S^{-1} and they observed increase of callus induction but reduced regeneration capacity by this method.

Acclimatization of *in vitro* developed plantlets was very low using compost mixture. Pavlović et al. (2010) also observed low acclimatization of propagated plantlets of cauliflower and savoy cabbage. Sretenović – Rajičić et al., (2006) have shown that sucrose content in the rooting medium could be an important factor in acclimatization. Pavlović et al. (2010) has confirmed the effect of higher content of sucrose in rooting medium on the acclimatization of plantlets by achieving successful acclimatization (average of 58%) of the plantlets rooted on 4% sucrose containing medium with compared to 30% of survival rate of plantlets on 2% sucrose containing medium. Munshi et al. (2007) could achieved 86% success in acclimatization of *B. oleracea* var. *capitata* using substrate; soil: compost 2:1 ratio.

As all reported previous studies emphasized the genotype dependency on *in vitro* organogenesis of *Brassica oleracea* (Bhalla and Weerd, 1999; Cardoza and Stewart, 2004; Akasaka-Kennedy et al., 2005; Yu et al., 2010; Pavlović et al. 2010; Mollika et al., 2011; Qamar et al., 2014; Gerszberg et al., 2015; Liu et al., 2015), during this study as well was clearly observed similar response.

After successful mutation induction and selection, it is necessary to evaluate the resistance capacity of developed resistant plants to live pathogen in the both greenhouse and field conditions to conform the stability of the mutational resistance developed in the plant. Identification of induced mutational resistance gene or genes through *in vitro* mutation induction would be an advantage for future studies. Furthermore, it is necessary to check the vegetative growth and phenotypic characteristics and head production for checking the influence of mutagenesis treatment on its other characters in addition to inducing *Fusarium* resistance. But in this study, this was not analyzed.

4.2. Conclusion

This study provides basic idea towards the possible application of *in vitro* chemical mutagenesis and *in vitro* selection by culture filtrate to develop induced novel *Fusarium* resistance in *Brassica oleracea* var. *capitata* plants for future application of this technique as a biotechnological approach to advance the Fusarium resistance breeding in cabbage.

In conclusion, study results showed that the direct organogenesis and use of hypocotyl (10 days old) and shoot tip (6 days old) explants are potential explants for *in vitro* mutagenesis of *B.oleracea* var. *capitata* with variations among six cultivars tested. But further studies are needed for optimization of suitable *in vitro* culture protocol having higher regeneration efficiency in each genotype, for optimization of mutagenesis treatment to achieve efficient desirable degree of mutations with higher survival percentage, for optimization of acclimatization protocol to achieve the best success in *in vitro* mutagenesis for *Fusarium* resistance development in *Brassica.*

BIBLIOGRAPHY

1. Ahioowalia BS. 1998. *in vitro* techniques and mutagenesis for the improvemnent of vegetatively propergated plants: Jain SM, Brar DS, Ahloowalia BS(Eds) *somaclonal variation and induced mutations in crop improvement,* Kiuwer Academic Publishers, Dordrecht, pp 293-309.
2. Akasaka- Kennedy Y, Yoshida H and Takahata Y. 2005. Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): The influence of silver nitrate and genotype. Plant cell Rep. 24. 649 654.
3. Amusa NA 2006. Microbiology produced phytotocxins and plant disease management. Afr. J. Biotechnol., 5. 405-414.
4. An XL, Cai YL, Wang JG., Wang GQ, Sun HY., 2003. Chemical mutagen and its application in plant breeding. Acta Agriculturae Nucleatae Sinica 17, 239-242.
5. Anderson EM. 1933. *Fusarium* resistance in Wisconsin Hollander Cabbage. J Agric Res 47.639–661.
6. Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, et al., 2004. Antagonistic interaction between abscisic acid and jasmonate-ethylene signalling pathways modulates defense gene expression and disease resistance in Arabidopsis. Plant Cell.16. 3460–3479.
7. Anderson, M. E., and J. C. Walker, 1935. Histological studies of Wisconsin hollander and Wisconsin ballhead cabbage in relation to resistance to yellows. J. Ag. Res. 50. 823–836.
8. Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J.2002. MAP kinase signaling cascade in Arabidopsis innate immunity. Nature. 415, 977–983.
9. Ayaz, F.A., Hayırlıoglu-Ayaz, S., Alpay-Karaoglu, S., Gruz, J., Valentova, K., Ulrichova, J., Strnad, M., 2008. Phenolic acid contents of kale (*Brassica oleraceae* L. var. *acephala* DC.) extracts and their antioxidant and antibacterial activities. Food Chem. 107.9–25.
10. Bacon CW. 1996. Production of fusaric acid by Fusarium species. Appl Environ Microb 62(11).4039–4043.
11. Beecher, C.W., 1994. Cancer preventive properties of varieties of *Brassica oleracea*. Am. J. Clin. Nutr. 59, 1166–1170.
12. Bhalla PL and Smith N. 1998. *Agrobacterium tumerfaciens* mediated transformation of cauliflower, *Brassca oleracea* var. *botrytis*. Molecular Breeding 4. 531- 541.

13. Bhalla PL and Weerd N. 1999. *In vitro* propergation of cauliflower, *Brassica oleracea* var. *botrytis* for hybrid seed production. Plant cell, Tissue and Organ culture. 56. 89- 95.
14. Bhan, A.K. and M.L.H. Kaul. 2003. Frequency andspectrum of chlorophyll-deficient mutations induced in rice after treatment with radiation and alkylating agents. Mutat. Res. 36.311–317
15. Bima P, Mensurati F, Soressi GP. 1994. Effect of Gibberellic Acid (GA3) and Micropropagation on Axillary Shoot Induction in Monostem Genotype (To-2) of Tomato (L. Esculentum Mill). Current issues in plant molecular and cellular biology. 22. 411- 416.
16. Biswas MK, Dutt M, Roy UK, Islam R, Hossain M. 2009, Development and evaluation of *in vitro* somaclonal variation in strawberry for improved horticultural traits. Scientia Horticulturae 122.409–416.
17. Blank LM. 1937. Fusarium resistance in Wisconsin all seasons cabbage. J Agr Res 55.497–510.
18. Bouizgarne B. 2006. Early physiological responses of Arabidopsis thaliana cells to fusaric acid: toxic and signaling effects. New Phytol 169(1).209–218.
19. Bozzini, A. and G.T.S. Mugnozza. 2003. Relative frequency of chlorophyll to morphological and sterility mutations induced in durum wheat by radiations and chemicals.Mutat. Res. 9.589–597
20. Brar DS, Jain SM. 1998. Somaclonal variation: mechanism and applications in crop improvement. In. Jain SM, Brar DS, Ahloowalia BS(Eds) somaclonal variations and induced mutations in crop improvement, Kluwer Academic publishers, Dordrecht, The Netherlands, pp15-38.
21. Brooks JD., Paton VG, Vidanes G., 2001. Potent induction of phase 2 enzymes in human prostate cells by sulforaphane. Cancer Epidemiol. Biomarkers Prev. 10 (9). 949–954.
22. Brunner H. 1995. Radiation Induced Mutations for Plant Se-lection. Plant Breeding Unit Joint FAO/IAEA Programme IAEA Laboratories, Seibersdorf.
23. Bughio HR, Asad MA, Odhano IA, et al. 2007.Sustainable rice production through the use of mutation breeding. Pakistan J Bot. 39.2457-2461.
24. Buiatti M, Ingram DS. 1991 Phytotoxins as tools in breeding and selection of disease resistant plants. Experientia 47.811–819.
25. Bulk RW .1991. Application of cell and tissue culture and *in vitro* selection for disease esistance breeding – a review Euphytica 56:.269-285.

26. Burbulis N, Kuprienė R and Blinstrubienė A. 2008. Callus induction and plant regeneration from somatic tissue in spring rapeseed (*Brassica napus* L.) biologija 54 (4). 258–263.
27. Byers, T., Perry, G., 1992. Dietary carotenes, Vitamin C and Vitamin E as protective antioxidants in human cancers. Annu. Rev. Nutr. 12, 139–159.
28. Cahill DM, Benett IJ, McComb AJ .1992. Resistance of micropropagated *Eucalyptus marginata* to *Phytophthora cinnamomi*. Plant Dis 76. 630-632.
29. Cao J, Shelton AM, Earle ED., 2008. Sequential transformation to pyramid two *Bt* genes in vegetable Indian mustard (*Brassica juncea* L.) and its potential for control of diamondback moth larvae. Plant Cell Reports 27, 479-487.
30. Cassells AC, Curry RF. 2001. Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tissue Organ Cult 64.145–157.
31. Cheney, G., 1950. Anti-peptic ulcer dietary factor. J. Am. Diet Assoc. 26, 668–672.
32. Chikelu Mba. 2013. *Review* Induced Mutations Unleash the Potentials of Plant Genetic Resources for Food and Agriculture. Agronomy 3. 200-231.
33. Cole SJ, Yoon AJ, Faull KF, Diener AC. 2014. Host perception of jasmonates promotes infection by *Fusarium oxysporum* formae speciales that produce isoleucine- and leucine-conjugated jasmonates. Mol Plant Pathol. 15. 589–600.
34. Constantin, M.J. 1984. Potential of *in vitro* mutation breeding for the improvement of vegetatively propagated crop plants. Induced mutations for crop improvement in Latin America, Vienna, Austria. International Atomic Energy Agency (IAEA) (Vienna, Austria). 15(21). 59–77.
35. Couteaudier Y, Alabouvette C. 1990. Survival and inoculum potential of conidia and chlamydospores of Fusarium oxysporumf. sp. lini in soil. Can J Microbiol 36:551–556.
36. Czene M, Harms-Ringdahl M. 1995. Detection of single-strand breaks and formamidoprymidine-DNA glycosylase-sensitive sites in DNA of cultured human fibroblasts. Mutat Res 336.235–242.
37. Czymmek KJ. 2007. In vivo time-lapse documentation using confocal and multi-photon microscopy reveals the mechanisms of invasion into the Arabidopsis root vascular system by *Fusarium oxysporum*. Fungal Genet Biol 44(10).1011–1023.
38. Da Silva TCR, Carvalho CR. 2014. Vertical heterogeneity of DNA ploidy level assessed by flow cytometry in calli of Passiflora cincinnata. In Vitro Cell Dev Biol Plant 50(2).158–165.

39. Daud NFA, Hasbullah NA, Azis NA, Rasad FM, Amin MAM, and Lassim MM. 2015. *In Vitro* Regeneration of *Brassica Oleraceae* L. Var *Capitata* through Stems, Roots, Leaves and Petioles Culture. International Conference on Agricultural, Ecological and Medical Sciences (AEMS-2015) April 7-8, 2015 Phuket (Thailand). http://dx.doi.org/10.15242/IICBE.C0415
40. Diener AC, Ausubel FM., 2005.Resistance to Fusarium oxysporum 1, a dominant Arabidopsis disease-resistance gene, is not race specific. Genetics.171.305–321.
41. Dong X, Xiong Y, Ling N, Shen Q, Guo S., 2014. Fusaric acid accelerates the senescence of leaf in banana when infected by Fusarium. World J Microbiol Biotechnol. 30. 1399–408. doi: 10.1007/s11274-013-1564-1. pmid:24282097
42. Dong XN. SA. 1996. JA, ethylene, and disease resistance in plants. Plant Biology. 1.316–323.
43. Donovan AM, Morgan R, Valombra-Piagnani C, Ridout MS, James DJ & Garrett CME. 1994. Assessment of somaclonal variation in apple. I Resistance to the fire blight pathogen, *Erwinia amylovora*. J. Hort. Sci. 69.105–113
44. Duncan RR (1997) Tissue culture-induced variation and crop improvement. Adv Agron 58.201–240.
45. Dunemann F and Grunewaldt J. 1990. In vitro Mutagenesis in *Brassica oleracea* var. *italica* Plenck (Broccoli). Die Gartenbauwissenschaft 55(4). 155-158.
46. Eapen S and George L. 1997. Plant regeneraton from peduncle segments of oil seed *Brassica* species: Influence of silver nitrate and silver thiosulfate. Plant cell, Tisue and Organ culture. 51. 229-22.
47. Edgar CI, McGrath KC, Dombrecht B, Manners JM, Maclean DC, Schenk PM, et al., 2006.Salicylic acid mediates resistance to the vascular wilt pathogen *Fusarium oxysporum* in the model host *Arabidopsis thaliana*. Aust Plant Pathol. 35.581–591.
48. Fang j. 2011. *In vitro* mutation induction of *Saintpaulia* using Ethyl Methane sulfonate. Horticulture 46(7). 981- 984.
49. Farahani F, Yari R, Masoud S.2011. Somaclonal variation in Dezful cultivar of olive (*Olea europaea* subsp. *europaea*). Gene Conserve 10.216–221.
50. Farnham MW, Keinath AP, Smith JP. 2001. Characterization of Fusarium yellows resistance in collard. Plant Dis 85.890–894.
51. Feng-Ian Z and Takahata Y. 1999. Microspore mutagenesis and *in vitro* selection for resistance to soft rot diseace in chinese cabbage (*Brassica compestris* L. ssp. *pekinensis*). Breeding Science 49. 161-166.

52. Gao X, Yang D, Cao D, Ao M, Sui X, Wang Q, Kimatu JN, Wang L. 2010. In vitro micropropagation of Freesia hybrid and the assessment of genetic and epigenetic stability in regenerated plantlets. J Plant Growth Regul 29.257–267.
53. Gao, H., C. H. Beckman and W. C. Mueller, 1995(a). The nature of tolerance to *Fusarium oxysporum* f. sp. *lycopersici* in polygenically field-resistant marglobe tomato plants. Phys. Mol. Plant Pathol. 46. 401–412.
54. Gao, H., C. H. Beckman and W. C. Mueller, 1995(b). The rate of vascular colonization as a measure of the genotypic interaction between various cultivars of tomato and various formae or races of *Fusarium oxysporum*. Phys. Mol. Plant Pathol. 46. 29–43.
55. Gerszbery A, Hnatuszko K, Kowalczyk T. 2015. *In vitro* regeneration of eight cultivar of *Brassica oleracea* var. *capitata*. N vitro cell. Dev.Biol-Plant. 51.80-87.
56. Glazebrook J. 2001. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens.Annual Review of Phytopathology 43. 205–222.
57. Hadi F and Fuller MP. 2013. Chemically Induced Mutants of *Brassica oleracea* var. *botrytis* Maintained Stable Resistance to Drought and Salt Stress after Regeneration and Micropropagation. American Journal of Plant Sciences, 4, 498-507.
58. Hammerschlag F.A. 1984. In vitro approaches to disease resistance. In: Advances in agricultural Biotechnology “Application of Genetic Engineering to Crop Improvement” (G.B. Collins, J.G. Petolino, Eds.). Martinus Nijhoff/Dr W. Junk Publishers,pp 453- 489.
59. Hammerschlag FA .1990. Resistance response of plants regenerated from peach callus cultures to *Xanthomonas campestris* pv. *pruni*. J Amer Soc Hort Sci 115. 1034-1037.
60. Handayani T. 2014. Regeneration of Broccoli (*Brassica oleracea* L var. *Bejo*) from hybrid mature seed and molecular analysis of regenerants. International conference on Agriculture and Biotechnology. 79.11.
61. Hare Krishna, Alizadeh M, Singh D, Singh U, Chauhan N, Eftekhari M, Kishan Sad R. 2016. Somaclonal variations and their applications in horticultural crops improvement Biotech 6.54.
62. Heath-Pagliuso S, Pullman J, Rappaport L .1988. Somaclonal variation in celery: screening for resistance to *Fusarium oxysporum* f. sp. *apii*. Theor Appl Genet 75. 446-451.
63. Heath-Pagliuso S, Pullman J, Rappaport L.1989. “UC-T3 somaclone”: celery germplasm resistant to *Fusarium oxysporum* f. sp. *apii,* race 2. HortSci 24. 711-712.
64. Huang YH, Hartman GL .1998. Reaction of selected soybean genotypes to isolates of *Fusarium solani* f. sp. *glycines* and their culture filtrates. Plant Dis 82. 999-1002.

65. Ingram DS, Helgeson JP. 1980. Tissue Culture Methods for Plant Pathologists. Blackwell Scientific Publications, Oxford, UK, pp 272. ISBN: 9780632007158.
66. Jacobs, M. 2005. Comparaison de l'action mutagen d'agents alkylants et des radiations gamma chez Arabidopsis thaliana. Radiat. Bot. 9. 251–268.
67. Jevremovic´ S, Subotic´ A, Miljkovic´ D, Trifunovic´ M, Petric´ M, Cingel A. 2012. Clonal fidelity of Chrysanthemum cultivars after long term micropropagation by stem segment culture. Acta Hortic 961.211–216.
68. Jones JD, Dangl JL., 2006. The Plant immune system. Nature: 16.323–9.
69. Jong-Yi F. 2011. In Vitro Mutation Induction of Saintpaulia Using Ethyl Methanesulfonate. Hortscience 46(7).981–984
70. Joyce SM, Cassells AC, Jain SM. 2003. Stress and aberrant phenotypes in vitro culture. Plant Cell Tissue Organ Cult 74.103–12.
71. Kaeppler SM, Kaeppler HF, Rhee Y. 2000.Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology; 43.179-188.
72. Kantoglu1 Y, Seçer E, Erzurum K, Tutluer I, Kunter B, Peşkircioğlu1 H, Sağel1 Z. 2010. Improving tolerance to *Fusarium oxysporum* f. sp. *melonis* in melon using tissue culture and mutation. Mass screening thechniques fr selecting crops resistant to disease. International atomic energy agency, Vienna. 235-244. ISBN 978-92-0-105110-3.
73. Karp A. 1994. Origins, causes and uses of variation in plant tissue cultures. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Springer, New York, 139-152.
74. Kashiwa T, Inami K, Fujinaga M, Ogiso H, Yoshida T, Teraoka T,Arie T. 2013. An avirulence gene homologue in the tomato wilt fungus Fusarium oxysporum f. sp. lycopersici race 1 functions as a virulence gene in the cabbage yellows fungus F. oxysporum f.sp. conglutinans. J Gen Plant Pathol 79.412–421.
75. Kassie, F., Parzefall, W., Musk, S., Johnson, I., Lamprecht, G., Sontag, G., Knasmuller, S., 1996. Genotoxic effects of crude juice from Brassica vegetables and juices extracts from phyto pharmaceutical preparations and spices of cruciferous plants origin in bacterial and mammalian cells. Chem.-Biol. Interact. 10. 1–16.
76. Kharkwal MC. 2012. A brief history of plant mutagenesis. Plant mutation breeding and biotechnology. Wallingford: pp. 21-30. ISBN 9781780640853.
77. Kidd BN, Kadoo NY, Dombrecht B, Tekeoğlu M, Gardiner DM, Thatcher LF, et al., 2011. Auxin signalling and transport promote susceptibility to the root-infecting fungal pathogen Fusarium oxysporum in Arabidopsis. Mol Plant Microbe Interact.24.733–748.
78. Knogge W. 1996. Fungal infection of plants. Plant Cell 8(10).1711.

79. Koike M, Nanbu K .1997. Phenylalanine ammonia-lyase activity in alfalfa suspension cultures treated with conidia and elicitors of *Verticillium albo-atrum*. Biol Plant 39. 349-353.
80. Koike M, Shimada T, Amemiya Y. 1993b. Alfalfa–*Verticillium albo-atrum* interactions IV. Reactions of alfalfa protoplasts to fungal culture filtrates and cell wall components. J Jap Soc Grass Sci 39. 101-107.
81. Krishna H, Sairam RK, Singh SK, Patel VB, Sharma RR, Grover M, Nain L, Sachdeva A. 2008. Mango explants browning: effect of ontogenic age: mycorrhization and pre-treatments. Sci Hortic 118.132–138.
82. Krishna H, Singh D .2013. Micropropagation of lasora (Cordia myxa Roxb.). Indian J Hortic 70.323–327
83. Kuanar A, Nayak PK, Subudhi E and Nayak S. 2014. *In Vitro* Selection of Turmeric Somaclone Resistant to *Fusarium oxysporum* f.sp. *Zingiberi*. Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci 84(4). 1077-1082.
84. Kuznetsova OI, Ash OA, Gostimsky SA. 2006. The effect of the duration of callus culture on the accumulation of genetic alternation in pea Pisum sativum L. Russ J Genet 42.555–562.
85. Larkin PJ, Scowcroft WR. 1983. Somaclonal variation and crop improvement, In. Kosuge T, Meredith C, Hollaender A (Eds) *Genetic engineering of plants,* PLENUM press, New York, pp 289-314.
86. Lebeda A and Švábová L. 2010. In vitro screening methods for assessing plant disease resistance MASS SCREENING TECHNIQUES FOR SELECTING CROPS RESISTANT TO DISEASES. 5-45. ISBN 978-92-0-105110-3
87. Lebeda A, Švábová L. 1997. Variation in response of several wild *Pisum* spp. to *Fusarium solani* and *Fusarium oxysporum*. Cereal Res Commun 25. 845-846.
88. Leva AR, Petruccelli R and Rinaldi LMR. 2012. Somaclonal Variation in Tissue Culture: A Case Study with Olive. Recent Advance in Plant *In vitro* Culture. Pp.123-150. ISBN 978953510787.
89. Liu XX, Lang SR, Su LQ, Liu X and Wang XF. 2015. Improved *Agrobacterum*-mediated transformation and high efficiency of root formation from hypocotyl meristem of spring *Brassica napus* “Precocity” cultivar. Genet. Mol. Res. 14(4). 16840- 16855.
90. Lv H, Fang Z, Yang L, Xie B, Liu Y, Zhuang M, Zhang Y, Yang Y. 2011.Research on screening of resistant resources to *Fusarium* wilt and inheritance of the resistant gene in cabbage. Acta Horticulturae Sinica., 38 (5).875-885.

91. Lv H, Wang Q, Yang L, Fang Z, Liu Y, Zhuang M, Zhang Y, Yang Y, Xie B and Wang X. 2014. Breeding of cabbage (*Brassica oleracea* L. Var. *Capitata*) with *Fusarum* wilt resistance based on microspore culture and marker-assistaed selection. Euphytica, 200. 465-475.
92. Lv H, Fang Z, Yang L, Zhang Y, Wang Q, Liu Y, Mu Zhuang, Yang Y, Xie B, Bo Liu, Liu J, Kang J and Wang X. 2014. Mapping and analysis of a novel candidate Fusarium wilt resistance gene FOC1 in Brassica oleracea. BMC Genomics 15.1094- 1104.
93. Lyons R, Stiller J, Powell J, Rusu A, Manners JM, Kazan K. 2015. Fusarium Oxysporum Triggers Tissue-Specific Transcriptional Reprogramming in Arabidopsis thaliana. PLoS ONE 10(4): e0121902. doi:10.1371/journal.pone.0121902
94. Maluszynski M, Ahlowalia BS and Sigurbjornsson. 1995. Application of *in vitro* and *in vivo* mutation techniques for crop improvement. Euphytic 85. 303- 315.
95. Mangal M and Sharma DR. 2002. In vitro mutagenesis and cell selection for the induction of black rot resistance in cauliflower. The Journal of Horticultural Science and Biotechnology 77(3). 268-272
96. Matsumura A, Nomitzu T, Furukani N, et al. 2010. Ray florets color and shape mutants induced by 12C5C ion beam irradiation in chrysanthemum. Scientia Horticulturae.123.558-561.
97. McCoy TJ. 1987. Characterization of alfalfa (*Medicago sativa* L.) plants regenerated from selected NaCl lines. Plant cell Reports 6. 417-422.
98. Metz TD, Dixit R, Earle ED., 1995. *Agrobacterium tumefaciens* mediated transformation of broccoli (*Brassica oleracea* var. *italica*) and cabbage (*Brassica oleracea* var. *capitata*). Plant Cell Reports 15. 287-292.
99. Miao S, Duncan DR, Widholm J. 1983. Selection of regenerable maize callus cultures resistant to 5methyl tryptophan, S2aminoethyl-L-cysteine and high levels of L-lysine plus Lthreonine. Plant cell, tissue and organ culture 14.3-14.
100. Miller OK, Hughes KW. 1980. Selection of paraquat resistants of tobacco from cell cultures. In vitro 16. 1085-1091
101. Mithem RF, Magrath R. 1992. Glucosinolates and resistance to *Leptosphaeria maculans* in wild and cultivated Brassica species. Plant Breed. 10. 60–68.
102. Mollika SR, Sarker RH and Hoque MI. 2011. *In vitro* plant regeneraton in *Brassica* spp. Plant Tissue Culture and Biotechnology. 21(2).127-134.

103. Munshi MK, Roy PK, Kabir MH and Ahmed G. 2007. *In vitro* regeneration of cabbage (*Brassica oleracea* L. Var. *Capitata*) through hypocotyl and cotyledon. Plant Tissue culture and Biotech, 17(2). 131-136.
104. Nagano AJ, Fukao Y, Fujiwara M, Nishimura M, Hara-Nishimura I. 2008. Antagonistic Jacalin-Related Lectins Regulate the Size of ER Body-Type β-Glucosidase Complexes in Arabidopsis thaliana. Plant Cell Physiol. 49(6). 969–980.
105. Ndagijimana V, Kahia J, Asiimwe T, Sallah PY, Waweru B, Mushimiyimana I, Ndirigwe J, Kirimi S, Shumbusha D, Njenga P, Kouassi M, Koffi E. 2014. *In vitro* effect of gibberellic acid and sucrose concentration on micropropergation of teo elite sweet potato cultivars in Ruwanda. Iternational Journal of Biotechnology and Molecular Biology Research. 5(1). 1-6.
106. Nicoloff, H. 2003. Effect of sodium acetate and sodium chloride on EMS-induced chlorophyll mutations in barley. Mutat. Res. 23.57–62.
107. Nieuwhof M., 1969. Cole Crops: Botany, Cultivation and Utilization. World Crops Series. Leonard Hill, London, UK, pp 353.
108. Nivas SK, DSouza L. 2014. Genetic fidelity in micropropagated plantlets of Anacardium occidentale L. (Cashew) an important fruit tree. Int J Sci Res 3.2142–2146.
109. Novak FJ.1991. In vitro mutation induction system in crop improvement. In: proceeding symposium Plant Mutation Breeding for crop Improvement, FAO/IAEA, Vienna2, 327-342.
110. Pantelides IS, Tjamos SE, Pappa S, Kargakis M, Paplomatas EJ., 2013. The ethylene receptor ETR1 is required for Fusarium oxysporum pathogenicity. Plant Pathol. 62. 1302–1309.
111. Pasqualetto, P. L., R. H. Zimmerman and I. Fordham. 1986. Gelling agent and growth regulator effects on shoot vitrification of `Gala' apple in vitro. J. Amer. Soc. Hort. Sci., 111. 976-980.
112. Pasqualetto, P. L., R. H. Zimmerman and I. Fordham. 1988. The influence of cation and gelling agent concentration on vitrification of apple cultivars in vitro. Plant Cell Tissue Organ Cult.,14. 31-40.
113. Patade VY, Suprasanna P,Kulkarni UG, Bapat VA .2006. Selection for abiotic (salinity and drought) stress tolerance and molecular characterization to tolerant lines in sugarcane. BARC newsletter 273. 244-257
114. Pathirana R. 2011. Plant mutation breeding in agriculture. Perspectives in Agriculture, Veterinary Science, Nutrition and Natural resources, 6(32).107-125.

115. Paul S, Sikdar S R., 1999. Expression of *npt* II marker and gus reporter genes and their inheritance in subsequent generations of transgenic *Brassica* developed through *Agrobacterium* mediated gene transfer. Current Science (India), 76, 1569-1573.
116. Pavlovíć S, Vinterhalter B, Mitić N, Adžić S, Pavlovíć N, Zdravković M and Vinterhalter D. 2010. *In vitro* shoot regeneration from seedling explants in *Brassica* vegetables, red cabbage, broccoli, savoy cabbage and cauliflwer. Arch. Biol. Sci. Belgrade, 62(2). 337-345.
117. Penna S, Vitthal SB, Yadav PV,. 2012. *In Vitro* Mutagenesis and Selection in plant tissue cultures and their prospects for crop improvement. Bioremediation, Biodiversity and Bioavalability 6(1). 6-14.
118. Pinet-Leblay C, Turpin FX and Chevreau E.1992. Effect of gamma and ultraviolet irradiation on adventitious regeneration from *in vitro* cultured pear leaves. Euphytica. 62. 225–233
119. Predieri S. 2001. Mutation induction and tissue culture in improving fruits. Plant Cell, Tissue and Organ Culture 64.185–210.
120. Prior RL., Cao G., 2000. Antioxidant phytochemicals in fruits and vegetables. Diet and health implications. Hortic. Sci. 35. 588–592.
121. Pu Z, Shimizu M, Zhang Y, Nagaoka T, Hayashi T, Hori H, Matsumoto S, Fujimoto R, Okazaki K. 2012. Genetic mapping of a *Fusarium* wilt resistance gene in *Brassica oleracea*. Mol Breed 30.809–818.
122. Qamar Z, Nasir IA, Jahangir GI and Husnain T. 2014. *In vitro* production of cabbage and cauliflower. Adv. Life Sci. 1(2).112-118.
123. Rask L, Andréasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J. 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol. 42(1).93-113.
124. Rego, L.V. and R.T. Faria. 2001. Tissue culture in ornamental plant breeding review. Crop Breed. Appl. Biot. 1.285–300.
125. Roy, M.K., Takenaka, M., Isobe, S., Tsushida, T., 2007. Antioxidant potential, antiproliferative activities, and phenolic content in water-soluble fractions of some commonly consumed vegetables: effects of thermal treatment. Food Chem. 103. 106–114.
126. Sahijram, L., Soneji, J.R., Bollama, K.T., 2003. Analyzing somaclonal variation in micropropagated bananas (Musa spp.). In Vitro Cell Dev. Biol. Plant 39 (6). 551– 556.
127. Sales EK, Butardo NG. 2014. Molecular analysis of somaclonal variation in tissue culture derived bananas using MSAP and SSRmarkers. Int J Biol Vet Agric Food Eng 8.63–610.

128. Saravanan S, Sarvesan R, Vinod MS. 2011. Identification of DNA elements involved in somaclonal variants of Rauvolfia serpentine (L.) arising from indirect organogenesis as evaluated by ISSR analysis. Indian J Sci Technol 4.1241–1245.
129. Sato M, Hosokawa M, Doi M. 2011b. Somaclonal variation is induced de novo via the tissue culture process: a study quantifying mutated cells in Saintpaulia. PLoS ONE 6. e23541.
130. Schenk PM, Kazan K, Rusu AG, Manners JM, Maclean DJ. 2005. The SEN1 gene of Arabidopsis is regulated by signals that link plant defense responses and senescence. Plant Physiol Biochem.; 43. 997–1005.
131. Shimizu M, Pu ZJ, Kawanabe T, Kitashiba H, Matsumoto S, Ebe Y, Sano M, Funaki T, Fukai E, Fujimoto R, Okazaki K. 2015. Map-based cloning of a candidate gene conferring Fusarium yellows resistance in Brassica oleracea. Theor Appl Genet. Jan; 128(1).119-130.
132. Singh S, Verma AK. 2015. A review on efforts of induced mutagenesis for qualitative and quantitative improvement of oilseed Brassicas. Journal of Pharmacognosy and Phytochemistry 4(2). 298-302.
133. Siragusa M, Carra A, Salvia L, Puglia A, De Pasquale F, Carimi F. 2007. Genetic instability in calamondin (Citrus madurensis Lour.) plants derived from somatic embryogenesis induced by diphenylurea derivatives. Plant Cell Rep 26.1289–1296.
134. Skirvin, R.M., Norton, M., Mcpheeters, K.D., 1993. Somaclonal variation: has it proved useful for plant improvement? Acta Hortic. 336. 333–340.
135. Slavov S. 2005. Phytotoxins and in Vitro Screening for Improved Disease Resistant Plants, Biotechnology & Biotechnological Equipment, 19(3). 48-55.
136. Slavov S., Blagoeva-Nikolaeva V. 1995. Effect of *Phytophthora parasitica* var. *nicotianae* (race 0) Culture Filtrate on Direct Organogenesis in Tobacco Stem Explants. Biotechnol. & Biotechnol. Eq., 9(2/3).15-22.
137. Smith, R., and J. C. Walker, 1930. A cytological study of cabbage plants in strains susceptible or resistant to yellows. J.Agric. Res. 41. 17–35
138. Smulders M, de Klerk G. 2011. Epigenetics in plant tissue culture. Plant Growth Regul 63.137–146.
139. Srejovi Srejović V. & Nešković, M. 1985. Effect of gibberellic acid on organogenesis in buckwheat tissue culture. Biol Plant (1985) 27: 432
140. Sretenović-Rajičić T, Ninković S, Miljuš-Dukić J, Vinterhalter B, Vinterhalter D.2006. *Agrobacterium rhizogenes*-mediated transformation of *Brassica oleracea* var. *sabauda* and *B. oleracea* var. *capitata*. Biol Plant. 50.525–530.

141. Sretenović-Rajičić T, Ninković S, Uzelać B, Vinterhalter B, Vinterhalter D. 2007. Effects of plant genotype and bacterial strain on *Agrobacterium tumefaciens*-mediated transformation of *Brassica oleracea* L. var. *capitata*. Russ J Plant Physiol. 54.653–658.
142. Sretenović-Rajičić, T., Ninković, S., Vinterhalter, B., MiljušĐukić J., and D. Vinterhalter (2004). Introduction of resistance to herbicide BastaR in Savoy cabbage. Biol.Plant. 48, 431-436.
143. Storti E, Latil C, Salti S, Bettini P, Bogani P, Pellegrini MG, Simeti C, Molnar A, Buiatti M. 1992. The *in vitro* physiological phenotype of tomato resistance to *Fusarium oxysporum* f. sp. *lycopersici*. Theor Appl Genet 84. 123-128.
144. Sun S, Zhong J, Li S, Wang X. 2013. Tissue culture-induced somaclonal variation of decreased pollen viability in torenia (Torenia fournieri Lind.). Bot Stud 54(1).36.
145. Takahashi H, Takatsugu T, Tsutomu M .1992. Gene analysis of mutant resistant to *Alternaria alternata* strawberry pathotype selected from calliclones of strawberry cultivar Morioka-16. J Jap Soc Hort Sci 61. 347-351.
146. Tanurdzic M, Vaughn MW, Jiang H, Lee TJ, Slotkin RK, Sosinski B,Thompson WF, Doerge RW, Martienssen RA. 2008. Epigenomic consequences of immortalized plant cell suspension culture. PLoS Biol 6(12). 2880–2895.
147. Telles-Pupulin AR. 1996. Effects of fusaric acid on respiration in maize root mitochondria. Biol Plantarum 38(3).421–429.
148. Thatcher LF, Gardiner DM, Kazan K, Manners JM., 2012. A highly-conserved effector in *Fusarium oxysporum* is required for full virulence on *Arabidopsis*. Mol Plant Microbe Interact.25.180–90.
149. Thatcher LF, Manners JM, Kazan K. 2009. *Fusarium oxysporum* hijacks COI1-mediated jasmonate signaling to promote disease development in *Arabidopsis*. Plant J. 58. 927–939.
150. Thatcher LF, Powell JJ, Aitken EA, Kazan K, Manners JM., 2012. The lateral organ boundaries domain transcription factor LBD20 functions in *Fusarium* wilt susceptibility and jasmonate signaling in *Arabidopsis*. Plant Physiol.160. 407–18.
151. Thompson JK. 2002. Yield evaluation of cabbage varieties. *J. Agric. Technol*., 5.15-19.
152. Trusov Y, Sewelam N, Rookes JE, Kunkel M, Nowak E, Schenk PM, et al., 2009.Heterotrimeric G proteins-mediated resistance to necrotrophic pathogens includes mechanisms independent of salicylic acid, jasmonic acid/ethylene and abscisic acid-mediated defense signaling. Plant J.58. 69–81.
153. Van den Bulk RW .1991. Application of cell and tissue culture and *in vitro* selection for disease resistance breeding – a review. Euphytica 56. 269–285.

154. Van Harten, A.M. 1998. Effect of gibberellic acid on organogenesis in buckwheat tissue culture. Mutation breeding: Theory and practical applications. Cambridge University Press. Cambridge, UK. pp. 59-77. 163-251. ISBN 0521470749.
155. Vardi A., Epstein E., Breiman A., 1986. Is the *Phytophthora citrophthora* culture filtrate a reliable tool for the *in vitro* selection of resistant citrus variants. TAG, 72. 569-574.
156. Velmurugan M, rajamani K, Paramaguru P, Gnanam R, Kannan Bapu JR, Harisudan C and Hemalatha P. 2010. *In vitro* mutation in horticultural crops a review. Agric. Rev, 31(1). 63-67.
157. Walker JC, Monteith J Jr, Wellman FL. 1927. Development of three mid-season varieties of cabbage resistant to yellows (*Fusarium conglutinans* Woll.). J Agric Res 35.785–810.
158. Walker JC. 1930. Inheritance of *Fusarium* resistance in cabbage.J Agr Res 40:721–745.
159. Wani MR, Kozgar MI, Tomlekova N, et al. 2014. Mutation breeding:a novel technique for genetic improvement of pulse crops particularly Chickpea (Cicer arietinum L.). Improvement of crops in the era of climatic changes. Sringer, New York.Vol. 2. pp. 217-248.
160. Watanabe H. 2001. Significance and expectations of ion beam breeding. Gamma Field Symposia.40.15-19.
161. Widoretno W, Harran S, Sudarsono. 2003. Variation in qualitative and quantitative characters among somaclones of soybean derived from in vitro selected somatic embryos. Hayati 10. 110-117.
162. Williams R. D. 1990. Black walnut. In: (Eds.): R.M. Burns; B.H. Honkala. Silvics of North America, vol. 2: Hardwoods. Agriculture Handbook 654. USDA Forest Service, USA, pp.391-400.
163. Xing, M., Lv, H., Ma, J., Xu, D., Li, H., Yang, L. Kang J, Wang X, and Fang, Z. 2016. Transcriptome Profiling of Resistance to Fusarium oxysporum f. sp. conglutinans in Cabbage (Brassica oleracea) Roots. PLoS ONE, 11(2), e0148048. http://doi.org/10.1371/journal.pone.0148048
164. Yildiz M, Er C. 2002. The effect of sodium hypochlorite solutions on in vitro seedling growth and shoot regeneration of flax (Linum usitatissimum). Naturwissenschaften 89. 259-261.
165. Yoder O.C. 1983. Use of pathogen-produced toxin in genetic engineering of plants and pathogens. Genetic engineering of Plants. 26. pp 335-353. ISBN: 9781468445466.
166. Yoder OC .1980. Toxns in pathogenesis. Annu.Rev. Phytopathol., 18.103-129.

167. Yu Y, Liu LS, Zhao YQ, Zhao B and Guo YD. 2010. A highly efficient *in vitro* plant regeneration and *Agrobacterium*-mediated transformation of *Brassica oleracea* var. b*otrytis*. New Zealand Journal of Crop and Horticultureal Sciece, 38. 235- 245.
168. Yun HE, Guanglong WAN, Zonglai JIN, Ling XU, Guixiang TANG, Weijun ZHOU. 2005. Mutagenic treatments of cotyledons for in vitro plant regeneration in oilseed rape. Biotechnology: Genomics and Its Applications 4.54-57.
169. Yusnita, Widodo, Sudarsono. 2005. In vitro selection of peanut somatic embryos on medium containing culture filtrate of *Sclerotium rolfsii* and plantlet regeneration. Hayati 12 (2).50-56.
170. Yusuff Oladosu, Mohd Y. Rafii, Norhani Abdullah, Ghazali Hussin, Asfaliza Ramli, Harun A. Rahim, Gous Miah & Magaji Usman. 2016. Principle and application of plant mutagenesis in crop improvement: a review, Biotechnology & Biotechnological Equipment, 301. 1-16, http://dx.doi.org/10.1080/13102818.2015.1087333
171. Zayova E, Vassilevska IR, Kraptchev B, Stoeva D. 2010. Somaclonal variations through indirect organogenesis in eggplant (Solanum melongena L.). Biol Divers Conserv 3.1–5.

LIST OF ABBREVATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
$AgNO_3$	Silver nitrate
AL	Cultivar: Albastros F1
BAP	6-Benzylaminopurine
cm	Centimeters
DC	Cultivar: DC 6
DMSO	Dimethylsulphoxide
DNA	Deoxyribo Nucleic acid
EMS	Ethyl methanesulfonate
g/L	Grams per Litter
GA_3	Gibberellic Acid
HCl	Hydrochloric acid
IBA	Indole-3-butyric acid
Ki	Kiklop F1
mg	Milligram
mg/L	Milligrams per Liter
mm	Millimeters
MS	Murasige and Skoog
NAA	Napthalene acetic acid

NaOCl	Sodium Hypochloride
NaOH	Sodium Hydroxide
$^{\circ}C$	Centigrade
PP	Pourovo pozdani
rpm	Revolution per minute
SD	Standard Deviation
Ta	Cultivar: Target F1
v/v	Volume to Volume
ZF	Cultivar: Zeus F1

APPENDICES

APPENDIX A: Constituents of the seed germination medium for *Brassica oleracea* var. *capitate*

MS medium (Murashige and Skoog Basal Salt Mixture) powder **4.33 g/L**

(Product from SIGMA ALDRICH Co. USA/ M5524[6])

Component consist in MS mxture;

Inorganic salts

NH_4NO_3	1650.0 mg/L
KNO_3	1900.0 mg/L
$MgSO_4$	180.7 mg/L
$MnSO_4.H_2O$	16.9 mg/L
$ZnSO_4.7H_2O$	8.6 mg/L
KH_2PO_4	170.0 mg/L
KI	0.83 mg/L
H_3BO_3	6.2 mg/L
$FeSO_4.7H_2O$	27.8 mg/L
$Na_2.EDTA$	37.26 mg/L
$CaCl_2.2H_2O$	332.2 mg/L

B5 Microelements

$Na_2MoO_4.2H_2O$	0.25 mg/L
$CuSO_4.5H_2O$	0.025 mg/L
$CoCl.6H_2O$	0.025 mg/L

Sucrose 10 g/L

Phytagel 7 g/L

pH 5.8

---

[6]< http://www.sigmaaldrich.com/technical-documents/protocols/biology/murashige-skoog.html#Basal Salts Mixtures> 10/05/2016

Appendix B: Constituents of the shoot induction medium for *Brassica oleracea* var. *capitate*

MS medium (Murashige and Skoog Basal Salt Mixture) powder	**4.33 g/L**
(Product from SIGMA ALDRICH Co. USA/ M5524)	

B5 Vitamins

Nicotinic acid	1 mg/L
Pyridoxine-HCl	1 mg/L
Thiamine-HCl	10 mg/L

Organic compounds

Myo-inositol	100 mg/L
Sucrose	20 g/L
Phytagel	7 g/L

Growth regulators

NAA – 0.5 mg/L

BAP - 3.0 mg/L

GA_3 – 0.01 mg/L

$AgNO_3$ – 0.5 mg/L

pH 5.8

Appendix C: Constituents of the callus induction medium for *Brassica oleracea* var. *capitate*

MS medium (Murashige and Skoog Basal Salt Mixture) powder	**4.33 g/L**
(Product from SIGMA ALDRICH Co. USA/ M5524)	

B5 Vitamins

Nicotinic acid	1 mg/L
Pyridoxine-HCl	1 mg/L
Thiamine-HCl	10 mg/L

Organic compounds

Myo-inositol 100 mg/L

Sucrose 20 g/L

Phytagel 7 g/L

Growth regulators

NAA – 0.5 mg/L

BAP - 3.0 mg/L

$AgNO_3$ – 0.5 mg/L

pH 5.8

Appendix D: Constituents of the shoot development medium for *Brassica oleracea* var. *capitate*

MS medium (Murashige and Skoog Basal Salt Mixture) powder 4.33 g/L

(Product from SIGMA ALDRICH Co. USA/ M5524)

B5 Vitamins

Nicotinic acid 1 mg/L

Pyridoxine-HCl 1 mg/L

Thiamine-HCl 10 mg/L

Organic compounds

Myo-inositol 100 mg/L

Sucrose 20 g/L

Phytagel 7 g/L

Growth regulators

NAA – 0.5 mg/L

BAP - 3.0 mg/L

GA_3 – 0.01 mg/L

pH 5.8

Appendix E: Constituents of the root induction medium for *Brassica oleracea* var. *capitate*

MS medium (Murashige and Skoog Basal Salt Mixture) powder	**4.33 g/L**
(Product from SIGMA ALDRICH Co. USA/ M5524)	
Sucrose	10 g/L
Phytagel	7 g/L

<u>Growth regulators</u>

IBA – 0.2 mg/L

pH 5.8

LIST OF FIGURES

Figure 1.1: Cabbage cultivating regions and regions of origination in the world……………..4

Figure 1.2: Distribution of mutant crop varieties by continents and percentage of mutant varieties have been officially released……………………………………………..10

Figure 2.1: Isolated *Fusarium oxysporium f.* Sp. *Conglutinans* on Czapek-Dox solid medium……………………………………………………………………….30

Figure 3.1: Seed germination and explant excision……………..……………………………34

Figure 3.2: Different responses of shoot induction and number of shoots producing by different explants with two different age groups of six different cultivars through direct organogenesis without mutagenesis treatment……………………………………38

Figure 3.3: Direct shoot induction from explants excised from 6 days old seedlings without mutagenesis……………………………………………………………………39

Figure 3.4: Different responses of shoot induction and number of shoots producing by different explants with two different age groups of six different cultivars through indirect organogenesis without mutagenesis treatment…………………………………..42

Figure 3.5: Indirect shoot induction from explants excised from 6 days old seedlings without mutagenesis…………………………………………………………………...43

Figure 3.6: Root induction of developed shoots and acclimatization of rooted plantlets in to natural environment…………………………………………………………..44

Figure 3.7: Analyzing optimum virulence concentration of *Fusarium* culture filtrate. 10 days old seedlings………………………………………………………………...48

Figure 3.8: Survival percentage of seedlings of different cultivars under different phytotoxic pressures of *Fusarium oxysporium f.* Sp. *Conglutinans* culture filtrate………..…49

Figure 3.9: Different responses of different explants of two different age groups of six different cultivars after mutagenesis treatment……………………………………………52

Figure 3.10: Direct shoot induction from hypocotyl and shoot tip explants excised from 6 days old seedlings…………………………………………………………………53

Figure 3.11: Selection of shoots having *Fusarium* resistance, developed after mutagenesis treatment…………………………………………………………………58

Figure 3.12: Selection of calli and induction of shoots carrying *Fusarium* resistance after mutagenesis treatment…………………………………………………………59

Figure 3.13: Development of resistance shoots from indirect organogenesis after mutagenesis and comparison between shoot induction without and with mutagenesis treatment………………………………………………………………..60

Figure 3.14: Root development and acclimatization of selected *Brassica* plantlets which supposed to carry *Fusarium* resistance………………………………………...61

LIST OF TABLES

Table 1.1: Common physical mutagens and their characteristics……………………………..16

Table 1.2: Commonly used chemical mutagens………………………………………………17

Table 1.3: *In vitro* selections for disease resistace in crop……………………………………21

Table 3.1: Average number of shoots per explant and percentage of total number of shoots developed by three different explants excises from 6 days and 10 days old seedlings of six cultivars after 45-60 days of culture initiation……………………………...37

Table 3.2: Average number of shoots per calli and percentage of total number of shoots developed by two different explants excises from 6 days and 10 days old seedlings of six cultivars after 45-60 days of culture initiation……………………………...41

Table 3.3: Percentage of root induction of *in vitro* produced shoots from different explants through direct and indirect organogenesis and acclimatization percentage of rooted plantlets of each cultivar…………………………………………………………45

Table 3.4: Phytotoxic effect of *Fusarium oxysporium f.* Sp. *conglutinans* culture filtrate on *in vitro* germinated *Brassica* seedlings…………………………………………...47

Table 3.5: Percentage of explants developing shoots and shoots per explants produced from two different explants (excises from 6 days and 10 days old seedlings) of six cultivars after mutagenesis treatment through direct organogenesis……………...51

Table 3.6: Selection of shoots developed from two types of explants with two different age after mutagenesis and root induction of selected *Fusarium* resistant shoots……...57

Table 3.7: Selection of treated calli and shoot induction after selection of mutated calli carrying resistance to culture filtrate……………………………………………………59