REPUBLIC OF TURKEY
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL SCIENCES AND TECHNOLOGIES

DEVELOPMENT OF IMI HERBICIDE RESISTANT BLACK CUMIN (*Nigella sativa* L) GENOTYPES

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Supervisor
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M. Sc. Thesis

January 2022
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SCIENTIFIC ETHICS SUITABILITY

I'm Ibrahim Ramzi HADI, hereby declare that this piece of work is the result of my research and that this work has never been submitted anywhere for any degree. All the sources of information used were obtained in accordance with academic ethics and have been duly acknowledged.

Ibrahim Ramzi HADI

COMPLIANCE WITH GUIDE

This master's thesis write up on the topic "Development of IMI Herbicide Resistant Black Cumin (*Nigella sativa* L.) Genotypes" was prepared with accordance Erciyes University's graduate Thesis Writing Directive

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ACKNOWLEDGEMENTS

All praise to Allah for this accomplishment and for giving me everything, and extend my sincere thanks to the Republic of Turkey for accepting me to study in one of its universities (Erciyes University).

Many thanks for Prof. Dr. Mehmet ARSLAN for this guidance, supporting and supervision during my master study period

I would also like to thank Dr. KAHRAMAN GÜRCAN and Mr. Ahmet SAY for their helping during my laboratory works.

Last but not least I would like to record my special thanks to my father and mother for their efforts in all steps of my life.

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Master Thesis, January 2022
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ABSTRACT

Weed species are a great threat to black cumin (*Nigella sativa* L.) cultivation due to their effective competition, reproductive and dispersal mechanisms. Weed control measures are not often practiced by black cumin growers. Therefore, seed yield reduction due to weed competition is inevitable in the black cumin production areas. Imidazolinone (IMI) resistant black cumin cultivars offer an alternative and an effective way to control the dominating weed species in the black cumin fields. IMI herbicides, which include imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz and imazaquin, successfully control a broad spectrum of grass and broadleaf weeds as well as weeds that are closely related to the crop by inhibiting the acetolactate synthase (ALS) enzyme. Post emergence application of IMI herbicide in resistant/tolerant black cumin cultivars can successfully expand limited options for weed control practices in black cumin fields. To develop IMI tolerant black cumin genotype, cotyledon and hypocotyl explants were *in vitro* cultured on 2,4-D and kinetin containing MS medium. A combination of 2,4-D and kinetin was found suitable for callus induction. The induced callus tissues were treated with 0.25, 0.50, 1.00, 2.00 and 4.00 µM imazamox. IMI herbicide resistant callus was obtained from the cotyledon and hypocotyl explants, however, none of the callus tissue developed into plantlet. Further studies are needed to obtain plant regeneration from the IMI treated black cumin callus tissues.

Keywords: Black cumin, IMI herbicide, *in-vitro* culture, *Nigella sativa*, resistant.

IMI GRUBU HERBİSİDE DAYANIKLI ÇÖREKOTU (*Nigella sativa* L.) GENOTİPİ GELİŞTİRME

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ÖZET

Yabancı ot türleri, etkili rekabet, çoğalma ve yayılma mekanizmaları nedeniyle çörek otu (*Nigella sativa* L.) yetiştiriciliği için büyük bir tehdit oluşturmaktadır. Yabancı ot kontrol önlemleri, çörekotu yetiştiricileri tarafından genellikle uygulanmamaktadır. Bu nedenle çörekotu ekim alanlarında yabancı ot rekabetinden dolayı verim azalışı kaçınılmazdır. İmidazolinon (IMI) grubu herbisitlere dayanıklı çörekotu çeşitleri, çörekotu tarlalarında hakim olan yabancı ot türlerini yok etmek veya kontrol etmek için alternatif ve etkili bir yol sunar. IMI grubu herbisitler olarak bilinen imazapir, imazapik, imazethapir, imazamox, imazametabenz ve imazaquin, asetolaktat sentaz (ALS) enzimini inhibe ederek geniş ve dar yapraklı yabancı otların kontrolünün yanı sıra kültür bitkisine yakın akraba yabancı otları da başarılı bir şekilde kontrol ederler. Dayanıklı/toleranslı çörekotu çeşitleri IMI grubu herbisitlerin çıkış sonrası uygulanmasına olanak sağlayarak, çörekotu tarlalarında yabancı ot kontrol uygulamalarındaki sınırlamaları ortadan kaldırabilir. IMI grubu herbisitlere dayanıklı çörekotu genotiplerini geliştirmek için kotiledon ve hipokotil eksplantları 2,4-D ve kinetin içeren MS ortamında kültüre alınmıştır. Kallus oluşumu için 2,4-D ve kinetin kombinasyonları en uygun hormon kombinasyonu olmuştur. Oluşan kallus dokularına 0.25, 0.50, 1.00, 2.00 ve 4.00 uM dozunda imazamox uygulaması yapılmıştır. Uygulamalar sonucunda IMI grubu herbisite dayanıklı kalluslar, kotiledon ve hipokotil eksplantlarından elde edilmiş, ancak kallus dokusunun hiçbirinden rejenerat bitki elde edilememiştir. IMI grubu herbisit uygulanmış çörekotu kallus dokularından rejenerant bitki elde etmek için daha ileri çalışmalara ihtiyaç olduğu sonucuna varılmıştır.

Anahtar Kelimeler: Çörekotu, dayanıklılık, IMI herbisit, *in vitro* kültürü

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INTRODUCTION

Black cumin (*Nigella sativa* L), an annual herbaceous plant species from Ranunculaceae family, is native to Southwest Asia, Southern Europe and North Africa and is commonly cultivated in the Mediterranean region, Eastern Europe, Middle East and Western Asia (Venkatachallam et al., 2010; Noor et al., 2012). The black cumin seeds are used generally for flavoring and treatment of various diseases in traditional medicine dating back to the ancient time (Ansari et al., 1988; Solmaz Mohammadi et al., 2017).

The black cumin seeds are known under different names such as Kalonji (India), Çörek otu (Turkey), Al-Habbah Al-Sawda or Habbat el Baraka (Arabic countries), Hak Jung Chou' in (China). Black cumin has been mentioned in several religious and ethnic books. The famous saying (Hadith) of Muslims prophet Mohammed that "black cumin is the remedy for all diseases except death which prompted many researchers to conduct several studies on it (Al-Ani, 2008; Ahmad et al., 2013; Tembhurne et al., 2014). Black cumin seeds are mentioned as the curative seeds in the holy bible and it is defined as the little black seed by Hippocrates and Dioscordes. The famous book of medicine by Ibn-Sina explained the significance of black cumin seeds (Paarakh, 2010; Tembhurne et al., 2014)). The black cumin seeds, used as a spice due to its bitter taste and characteristic aroma, contain 36-38% fixed oils, 0.4-2.5% essential oil, proteins, alkaloids and saponin (Ali and Blunden, 2003). Beside its culinary usage, it is also commonly used in folk medicine to treat asthma, bronchitis, cough, eczema, rheumatism, fever, headache and influenza for more than 2000 years (Burits and Bucar, 2000; Forouzanfar et al., 2014; Gharby et al., 2015).

Turkey has various and favorable agro ecological zones for production of numerous medicinal and aromatic plants. In Turkey, there are roughly 15 species of *Nigella* (Kökdil et al., 2005). The estimated black cumin production is more than 200 tones in

Turkey. Black cumin seeds are used as spice and seasoning for foodstuffs like bakery products and pickles among Turkish people.

The biological activities of black cumin are well studied and well-known. Black cumin has anti-inflammatory properties, reduces sugar and cholesterol level in the blood (Bingham, 1978), acts against stroke, relaxes the central nervous system and anti-tumorous (Worthen et al., 1998; De Tommasi et al., 2000). The major black cumin producing countries are India, Turkey, Bangladesh, China, Indonesia, Pakistan and Ethiopia.

Among different biotic stress factors responsible for lower black cumin yields, weed interference is of major importance. Weed interference in black cumin can cause up to 85% yield reduction due to its relatively open canopy and slow growth (Iqbal et al., 2011).

If high yields and good quality are to be achieved in black cumin production system, effective weed control is needed. Initially, hand weeding dominated most weeding practices, but it was gradually replaced with chemical control. Herbicide usage in the world has been increased 15-fold after the introduction of transgenic herbicide tolerance cultivars which offer the opportunity of controlling weeds through need-based applications of herbicides (Benbrook, 2016).

Weeds minimize crop production and degrade crop quality directly. Weeds significantly decrease crop yield. They take up nutrients, water, and moisture from the soil, as well as competing for light and oxygen, which has a detrimental impact on the crop. They restrict crop production by restricting the number of plants that can be planted (Rekha et al., 2012).

The Imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz, and imazaquin are imidazolinone herbicides that regulate weeds by inhibiting acetolactate synthase (ALS). In plants, ALS is needed for the biosynthesis of branched-chain amino acids. Mutagenesis and selection led to the discovery of several variant ALS genes conferring imidazolinone resistance in plants.

In all crops where weed competition for nutrients is a problem, critical seed growth cycles are critical, this is why the primary goal of this study is to develop imidazolinone (IMI) group herbicide-resistant black cumin (*Nigella sativa* L) variants through plant tissue culture technique and cell selection under in-vitro conditions.

CHAPTER 2

LITERATURE REVIEW

Black cumin (*N. sativa* L.), a dicotyledon of the Ranunculaceae family, is an impressive herb with a rich historical and religious history among the promising medicinal plants (Goreja, 2003). It has white or pale to dark blue flowers on a bushy, self-branching stem. Black cumin reproduces by producing a fruit capsule containing several white trigonal seeds. When the fruit capsule matures, it opens up, exposing the seeds inside, which become black when exposed to the air (Schleicher and Saleh, 2000).

The plant grows up to 20 to 30 cm tall, with finely divided, linear leaves. The flowers are small and delicate, with 6 to 12 petals and a pale blue and white color scheme. The fruit is a big, inflated capsule made up of three to seven joined follicles, each bearing a large number of seeds (Ismail et al., 2008).

Traditionally, the seeds and their oil have been used to treat many diseases. Herbal seeds are bitter and pungent and are used as an appetizer, and other traditional uses like diuretic, emmenagogue, galactagogue, thermogenic, carminative, anodyne, constipating, deodorant, anthelmintic, acrid, digestive, sudorific, expectorant, febrifuge, purgative, and abortifacient. Also black cumin is used to treat things like ascites, cough, jaundice, hydrophobia, and flu (Warrier, 1993; Sharma et al., 2005)

The phytochemical researches on black cumin are very limited. According to the reports, the black cumin fatty and volatile oil components of seeds were nigellone, nigellimine, nigellicine, avenasterol-5-ene, nigellimine-N-oxide, avenasterol-7-ene, campesterol, cholesterol, 24-ethyl-lophenol, citrostadienol, cycloeucalenol, gramisterol, lophenol, 243-methyllophenol, obtusifoliol, sitosterol, stigmastanol, stigmasterol, stigmasterol-7-ene, oleic acid, beta-amyrin, butyrospermol, esters of unsaturated fatty acids of C15 and higher terpenoids, dehydrostearic and linoleic acid esters, aliphatic

alcohol, taraxerol, tirucallol, nigellidine, carvone, d-limonene, cymene, hederagenin glycoside, melanthin, melanthigenin, bitter concept, tannin, resin, proteins, reducing sugar, glycosidal saponin, unsaturated hydroxyl ketone (Rastogi and Mehrotra, 1993; Sharma et al., 2005).Volatile oil contains trans-anethole (38.3%), p-cymene (14.8%), limonene (4.3%), and carvone (4.0%) (Nickavar et al., 2003), 4-benzenediol,2-(2-methoxypropyl)-5-methyl-1, thymol and carvacrol (Enomoto et al., 2001). Vanillic acid was said to be present in the root and shoot (Bourgou et al., 2008). The seed fatty oil contains cholesterol, campesterol, stigmasterol, β-sitosterol, α-spinasterol, (+)-citronellol, (+)-limonene, p-cymene, citronellyl acetate, carvone (Rastogi and Mehrotra, 1993), nigellone, arachidic, linolenic, linoleic, myristic, oleic, palmitic, palmitoleic and stearic acids. Fixed oil contains linoleic acid (55.6%), oleic acid (23.4%) and palmitic acid (12.5%).

A lot of studies on the effects of black cumin extracts or oil on various bacterial isolates has been reported. The extract and oil have been shown to have a wide range of antimicrobial action against a variety of bacteria. In vitro antibacterial activities of the essential oil, for example, exhibited strong action against numerous species, including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Shigella niger*, and *Vibrio cholera*, even at 1:100 dilutions (Agarwal et al., 1979). In general, the oil was more efficient against Gram-positive bacteria than Gram-negative bacteria. Antifungal activity was also discovered in the oil, notably against *Aspergillus species* (Agarwal et al., 1979).

The essential oil of black cumin was found to be efficient against Gram-positive (*Staphylococcus aureus, Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using the plate diffusion method. When *Bacillus subtilis* was employed, the antibacterial action was at its peak (El-Kamali et al., 1998).

Both the crude alkaloid extract and the aqueous extract of the seeds have been found to be effective against a range of pathogens (derived from human patients with septic arthritis), including those resistant to antibiotics (Morsi, 2000). Gram-negative isolates were impacted more than Gram-positive isolates, and the extracts, like several antimicrobial drugs, had more antibacterial activity at lower concentrations than at

higher ones. This might be due to physicochemical variables like solubility and diffusion.

Intraperitoneally administered bacteria oil has been found to be effective against murine cytomegalovirus infection in mice. It was claimed that the activity was due to the potentiating effect of black cumin oil on innate immunity (Salem and Hossain, 2000).

Both the fixed oil of *N. sativa* and thymoquinone (the major component of the essential oil) have been demonstrated to prevent non-enzymatic lipid peroxidation in liposomes (Houghton et al., 1995). Compounds extracted from black cumin (including thymoquinone, carvacol, t-anethole, and 4-terpineol) have also been found to exhibit significant free radical scavenging capabilities using thin-layer chromatography (TLC) (Burits and Bucar, 2000). In a number of further in vitro studies, these compounds were found to exhibit varied antioxidant activities but no prooxidant characteristics. The various chemicals in the oil were discovered to operate synergistically (that is, in a way that is greater than the sum of their separate effects). This emphasizes the significance of using the seeds' whole oil (or crude extract) in pharmacological investigations. This characteristic has already been observed in a variety of spices (Beckstrom-Sternberg and Duke, 1994). Although antioxidant action of black cumin is multifaceted, it does not appear to include iron-complexing activity (Burits and Bucar, 2000). Many human illnesses and disorders may be caused, at least in part, by the production of free radicals. As a result, antioxidant activity of black cumin might explain its folk medicine claims. This antioxidant activity might explain its ability to protect the liver from CCl4 poisoning (Nagi et al., 1999).

2.1. Herbicide

Crops differ in competitive ability with weeds. Weed competition of black cumin is very week. Among the factors which adversely affect the water and nutrient uptake, growth, yield and quality of black cumin, weed infestation is the worst one, since it has relatively open plant canopy and slow growth rate at the early growth stages. Black cumin fields are infested by variety of weed species belonging to different plant families (Table 2.1). In general, black cumin is less competitive compared with many crops. Therefore, weed interference is a major factor affecting black cumin production (Nadeem et al. 2013). The black cumin crop suffers severely due to heavy weed

infestation. Weed competition is more severe during the early stage growth stages. It has been estimated that weed competition during the growing periods would reduce black cumin seed yield up to 69% (Abrar et al., 2009). Hand weeding in the large production areas is very tedious and labor cost application and other mechanical weed control practices are not easy to apply due to narrow row spacing of this crop. Chemical weed control is much more meaningful for weed control in black cumin fields. However, there is not any herbicide available to control broad range of weed species after black cumin emergence. Therefore, development of herbicide resistant/tolerant black cumin is crucial for an effective weed control.

2.2. Herbicide resistant crop development methods

2.2.1. Conventional breeding

Under field conditions screening of crop cultivars to develop herbicide resistance has been carried since the use of herbicide in the production areas. The main objectives were of these studies were to develop herbicide resistant cultivars that the herbicide could be used safely to control weeds. Genetic variation in crops is a useful source of herbicide resistance or tolerance. Conventional breeding programs are quite successful when a few genes are responsible for herbicide resistance/tolerance. Herbicide resistance was generally caused by a change in a single gene in both weeds and crop plants, according to research. (Darmency, 1994), which makes it possible to breed resistant/tolerant herbicide-resistant cultivars in a short time (McGuire and Thurling, 1988). *Brassica campestris* was a good example for successful conventional breeding program to transfer atrazine resistance gene to other species of *Brassica* like canola, rapeseed and broccoli (Beversdorf et al., 1980; Machado and Bandeen, 1982; Souza Machado and Hume, 1987). Traditional breeding usually necessitates a comprehensive inspection of a massive number of plants, as well as a substantial amount of effort spent on selection and backcrossing. Conventional breeding program it is so hard to handle a high number of plants in the field and apply uniform herbicide exposure and selection pressure on plants.

2.2.2. Mutation Breeding

Mutation is a natural phenomenon that generates new variants of genes. Mutation is the main source of all genetic variations existing in crop plants. Mutation breeding can be called as the purposeful application of mutagens on plants to create genetic variations. Chemical and physical mutagens are considered good resolution for development of herbicide tolerant crop. Herbicide resistance crops can be developed by mutation breeding. Crop seeds are good plant material used for treatment of physical and chemical mutagens. Ethylnitrosourea and ethyl methanesulfonate are frequently used chemical mutagens. Artificial mutation is observed generally following self-fertilization of the first generation (Ml plants). Cross-fertilizing crop species require organized pollination when producing M2 generation (Roden and Woosley, 1983). Application of mutagens causes physiological injuries that often reduce fertility, germination and seedling growth in Ml plants. Despite these risks, a number of herbicide-resistant mutants, such as wheat, have been successfully produced in crops. (Newhouse et al. 1992), canola (Conner et al., 1994) and soybean (Sebastian and Chaleff, 1987; Sebastian et al., 1989).

Herbicide-resistant crops, such as soybean, have been developed by the application of physical and chemical mutagens (Sebastian et al., 1989 Sebastian and Chaleff, 1987), canola (Conner et al., 1994) and wheat (Newhouse et al., 1992).

2.2.3. The fusion of Protoplast

Plant protoplasts are important for somatic cell fusion and ne cultivar development. Development of crop cultivars for herbicide resistance through protoplast fusion and plant regeneration is becoming important due to somatic hybridization and the potential transformation of protoplast (Oxtoby and Hughes, 1989; Umate et al., 2005). Until first successful protoplast fusion of *Nicotiana*, it was impossible to hybridize two unrelated plant species (Carlson et al., 1972). Parasexual hybridization is a technique for fusing isolated protoplasts from two different parental species. Protoplast fusion has a number of advantages, including the transfer of nuclear genes between species and the formation of cybrids (cytoplasmic hybrids) that are hard to achieve by regular breeding (Conner and Meredith, 1989). To transmit cytoplasmically-inherited herbicide resistance, protoplast fusion and regeneration procedures can be exploited, especially

when employing interspecific hybrids. Datta et al. (1992) researched on Terbutryn-resistant plastids from the TBR2 mutant of *Nicotiana plumbaginifolia*. They introduced Terbutryn-resistant plastids into *Nicotiana tabacum* by protoplast fusion following X-irradiation of TBR2 protoplasts. The cybrid plants obtained by protoplast fusion were resistant to higher dosage of atrazine (10 kg/ha).

By transferring chloroplasts that contain herbicide resistance, the advantage of protoplast fusion was identified with the introduction of herbicide-resistant crops. Even though, it was possible to develop new herbicide resistant crop cultivars, the effectiveness of protoplast fusion is largely dependent on a number of parameters, including ideal culture conditions, protoplast maintenance, and protoplast callus induction followed by plant regeneration.

Table 2.1. Examples of protoplast fusion to develop triazine-resistant crops.

Crops used in protoplast fusion		Reference
Resistant Donor	Resistant Recipient	
Solanum nigrum	*Solanum tuberosum*	(Gressel, 1984)
Solanum nigrum	*Lycopersicum esculentum*	(Jain et al., 1998)
Brassica campestris	*Brassica ojeracea*	(Robertson et al., 1985)
Brassica campestris	*Brassica napus*	(Jourdan et al., 1989)

2.3. Engineering of Herbicide Resistance in Crop Plants

Herbicide-resistant crops or herbicide-tolerant crops can be developed by transgene technology or selection resistant mutants in cell or tissue culture. Use of biotechnology and molecular genetics allows the creation of genetic variation in crops and enables the transfer of desirable genetics from one species to another species. Transferring herbicide resistance genes are a remarkable commercial success in agriculture. Transferring herbicide resistance gene was the first agronomic trait using by by genetic manipulation (Comai et al., 1985). The emergence genetically engineered herbicide resistant crops made it possible for growers to use broad spectrum herbicides, glufosinate and glyphosate, to control all growing weed species that was previously impossible. Herbicide resistance genes have been successfully transmitted into plants using

agrobacterium-based vectors or direct DNA transfer into plant cells or protoplasts. Herbicide-resistant crops are developed in three ways using genetic engineering.

2.3.1. Over expression of herbicide resistant genes

Herbicide resistance is improved by having a high copy number of herbicide resistant genes and a high promoter activity of herbicide resistant genes as a result of overexpression of genes. An overexpression approach was used to produce resistance to herbicides such as phosphinothricin, glyphosate, and sulfonylurea (Shah et al., 1986; Eckes et al., 1989; Odell et al., 1990).

2.3.2. Herbicide detoxification

Herbicide resistant crops have higher and complete detoxification of herbicides than occurs in herbicide sensitive crops. Another strategy for preventing herbicide resistance in crops is to transfer herbicide detoxifying genes. Detoxification genes are mostly isolated from microorganisms. While there have been no reports of detoxifying genes for AHAS inhibitors, this strategy has been employed for other herbicides (De Block et al. 1987; Stalker et al., 1988; Buchanan-Wollaston et al., 1992).

2.3.3. Altered target site

A changed target site, i.e. a modified target protein with lower affinity for the herbicide, can be used to create herbicide resistance (Table 2.2). Herbicide resistance at the target site has been thoroughly examined (Maneechote et al., 1994; Eberlein and Devine, 1997; Shukla and Devine, 2000; Mallory-Smith and Preston , 2001). Some herbicides with major sites of action, such as those that inhibit acetyl-CoA carboxylase, acetolactate synthase, photosynthetic electron transport at photosystem II, and tubulin polymerization, have reported altered target sites. It appears to be more appropriate for gene transfer after herbicide resistance is developed via a gene encoding for an altered target site. This is a well-known technique for cultivating herbicide resistance in plants (Comai et al. 1985; Chuong et al. 1988; Haughn et al. 1988).

Table 2.2. Herbicides' molecular targets and resistance mechanisms

Suppressed process/molecular target	**Chemical class of herbicides**	**Mechanism of Resistance**
Synthesis of amino acids		
ALS; branched-chain amino acids	*Imidazolinones*	
	Chlorimuron	TSR and NTSR
	Imazaquin	TSR
	Pyrimidinyl benzolates	
	Chlorsulfuron	TRS and NTSR
	Imazethapyr	TSR and NTSR
	Benzulfuron	TSR and NTSR
	Sulfonylureas	
	Bispyribac	TSR and NTSR
	Penoxsulam	NTSR
	Triazolopyrimidine	
Glutamine synthase	Glufosinate	NTSR
EPSPS; shikimate pathway function	Glyphosate	TSR and NTSR
Auxin mimics		
F-box proteins	*Phenoxycarboxylates*	
	Dicamba	TSR and NTSR
	2,4-D	TSR and NTSR
	Picloram	TSR
	Picolinates	
	Quinclorac	NTSR
Synthesis of Carotenoid		
HPPD	Isoxaflutole	NTSR
	Tembotrione	NTSR
	Mesotrione	NTSR
	Triketones	
Lycopene cyclase	Amitrole	NTSR
Phytoene desaturase	*Diphenyl heterocycles*	
	Norflurazon	TSR
	Diflufenican	No resistance
	Beflubutamid	No resistance

Suppressed process/molecular target	**Chemical class of herbicides**	**Mechanism of Resistance**
	Picolinafen	No resistance
	Phenyl ethers	
	N-Phenyl heterocycles	
	Fluridone	TSR
Deoxy-D-xylulose phosphate synthase	Clomazone	NTSR
Cell division		
α-Tubulin	*Dinitroanilines*	
	Trifluralin	TSR
	Oryzalin	TRS
Synthesis of Chlorophyll		
PPO	*Diphenyl ethers*	
	Fomesafen	TSR
	Lactofen	NTSR
Synthesis of Fatty acid		
ACCase	*Aryloxyphenoxypropionates*	
	Tralkoxydim	TSR and NTSR
	Clodinofop	NTSR
	Fenoxaprop-P-ethyl	TSR and NTSR
	Sethoxydim	TSR
	Diclofop-methyl	TSR and NTSR
	Quizalofop	TSR
	Cyclohexanediones	
	Pinoxaden	NTSR
Very long-chain fatty acid synthases	Alachlor	NTSR
Fatty acid thioesterase	Metolachlor	NTSR
	Chloroacetamides	
	Flufenacet	NTSR
	Thiobencarb	NTSR
	Cinmethylin	NTSR
Photosynthesis		
Photosystem II D1 protein	*Amides*	
	Atrazine	TSR and NTSR

Suppressed process/molecular target	Chemical class of herbicides	Mechanism of Resistance
	Ureas	
	Bentazon	NTSR
	Triazines	
	Propanil	TSR and NTSR
	Chlorotoluron	NTSR
	Nitriles	
	Bromoxynil	NTSR
Photosystem I energy diversion	Paraquat	NTSR

NTSR: non-target-site resistance ; TSR: target-site resistance

Herbicides are substances that can destroy or slow the growth and development of plants. Herbicides are made up of more than 200 active compounds that have been registered all over the world.

Herbicides are categorized in a variety of ways, including by application timing (e.g., pre or post application), crop (e.g., corn herbicide), path of mobility in the plant (e.g., phloem, xylem, or both), chemical family (e.g., sulfonylureas, dinitroanilines), and mode of action (e.g., photosystem II inhibitors, ALS inhibitors).

Recent advancements in gene technology have been enabled the introduction and expression of herbicide resistant genes to control problem weeds with the application of broad spectrum herbicides. The AHAS molecule's biochemical basis has been thoroughly investigated (Yadav et al., 1986; Mazur, 1985). The bacterial AHAS enzyme contains 2 enzyme sub-units. However, The plat AHAS enzyme is made up of just one subunit and is found in chloroplasts (Miflin 1974). Therefore, the transferring bacterial genes required the expression of the two proteins from the sub-units, and the translocation of the enzyme in chloroplasts of the plant. As a result, plant AHAS genes were used to create transgenic AHAS inhibitor-resistant crops (Mazur et al., 1987; Lee et al., 1988; Haughn et al., 1988).

2.4. Cell culture approach for development of herbicide resistance

Plant cell and tissue cultures are the simplest biotechnological methods for development of herbicide resistant crops. Heritable genetic alterations in plant genomes for herbicide

resistance can be induced by somaclonal modifications in vitro. At the present time isolated plant cells or tissues have been used to develop herbicide resistant crops. In recent years, the use of cell and tissue cultures to discover potentially valuable agronomic features in plants has gained widespread acceptance. One of the primary advantages of these techniques is the ability to select new phenotypes from a physiologically and developmentally homogeneous population of cells cultivated in vitro. Plant cell and tissue cultures were originally used for micro-propagation of the crops. Later, it was also used to select useful traits such as resistant herbicide and altered environments. A special emphasis was given to develop herbicide resistant crops (Table 2.3). Herbicide-resistant cells can be studied in vitro under controlled conditions with a large number of cells in a separate culture medium, allowing for quick data collection and experiment repeatability (Gressel, 1979). BASF Inc. developed a sulfonylurea and sethoxydim herbicides resistant corn hybrid by using cell culture procedures. Following continual cultivated corn embryo tissues under strong sulfonylurea and sethoxydim selection pressure, they discovered a mutant cell line. The enzyme acetolactate synthase in the regenerated plant from the mutant cell line was resistant to sulfonylurea and sethoxydim herbicides (Zambrano et al., 2003). In liquid media containing 0.8 mM glyphosate, researchers established a glyphosate-tolerant sugar cane cell line. The regenerated plants had certain degree of tolerance up to 5-fold concentration of glyphosate. Caretto et al. (1994) developed sulfonylurea and chlorsulfuron herbicide resistant carrot cells and plant regenerations. Amplification of the acetolactate synthase gene caused resistance in regenerated plants. The incidence of genetic variation, determining selection methods, and evaluating the stability of selected cell lines are all important aspects to consider in plant tissue and cell culture when developing herbicide resistant crops.

The black cumin plant shows an excellent example in the tissue culture, hypocotyl, leaves, cotyledon and cotyledon node have the ability to grow and form callus. Also black cumin explants have a great respond to known plant growth regulator (Banerjee and Gupta 1974).

Chand and Roy (1980) compared leaf and stem explants of black cumin callus induction, they found that the response of leaf explant for callus induction was rapid than that of the stem explants. They concluded that the leaf explants could be a good

source for callus induction. On the other hand, the callus formation was induced from leaf, stem and root of black cumin on MS media containing NAA with coconut milk or MS with Kinetin and 2,4-D showed that the stem tissue was the best for callus formation (Banerjee and Gupta 1974; Suresh Chand and Roy 1980; Aziz et al., 1998).

Aziz et al. (1998) compared the ability of leaf, roots and stem parts of black cumin for callus formation when cultured on Murashige and Skoog media containing different concentrations of Kinetin and 2,4-D. Leaf and stem cuttings cultivated on MS with 10-6M 2,4-D and 510-6M Kinetin, respectively, produced the greatest callus induction.

Rasheed (2003) compared root, leaf and stem and explants of black cumin for callus formation on MS media containing 2,4-D (0.2 mg/L and 0.3 mg/L) and Kinetin (0.1 mg/L, 0.3 mg/L and 0.5 mg/L) and. The root explants showed the greatest callus induction.

El-Mahrouk et al. (2018) developed haploid plants through in vitro ovule cultures of black cumin. The tested media were Murashige and Skoog (MS) medium supplemented with different concentrations of kinetin (Kin), 6-benzyladenine (BA), naphthalene acetic acid(NAA) and 2,4-dichlorophenoxyacetic acid(2,4-D) and at (0, 0.5, 1, and 2) mg·LL1. Among the tested growth regulators of the plant, 2,4-D at 2 mg·LL1 had the best results for direct gynogenesis production and induction of haploids.

Genetic diversity occurs among somaclones in plant tissue and cell cultures are termed somaclonal variation. This kind of genetic variations include aneuploids, and morphological variants of economically important traits in the crop plants. The somaclonal variation term was commended by (Scowcroft and Larkin, 1981) for any variation in genetics identified in plant cell and tissue cultures: Incorporation of economically important traits created by somaclonal variantions can help for a quick success of superior cultivar improvement (Racchi et al., 1997; Jain, 1997). The occurrence probability of somaclonal variations can be affected by many factors such as explants origin, genotype of the explant, hormone compositions, numbers of subcultures and conditions of cultures. Effective selection and isolation of plant cells resistant to specific herbicides and regeneration of resistant plants depends on the availability of desirable genetic variations in the plant cells and tissue cultured in vitro. The utility of genetic variety was first demonstrated when disease resistant plants in potato and

sugarcane were recovered (Heinz and Mee, 1971). Somaclonal variations are complex phenomenon that occurs from an array of cellular and genetic mechanisms. Karyotypic changes, chromosomal rearrangement, chromosome aberrations, sister chromatid exchange, cryptic somatic crossing-over, transposable exchange, epigenetic variation in regenerated plants, variation in cytoplasmic organelle DNA and amplification and deletion in DNA sequences are the major fundamental measures donating to somaclonal variations (Scowcroft and Larkin, 1982; Larkin and Scowcroft, 1983). Somaclonal mutations are heritable mutations that occur in plant cell or tissue cultures (Scowcroft and Larkin, 1981; Scowcroft et al., 1985; Bettini and Buiatti, 1998; Wright and Penner, 1998; Wright et al., 1998). It was suggested that these kinds of mutations happen constantly in cell and tissue cultures. Longer cell culture cycles had accumulated more genetic variation in callus, tissue, protoplasts and cell suspension cultures (Lorz and Brown 1986; Oono, 1981; Hoffmann et al., 1982). Thus, the resistant could be occurred in plant cell populations before herbicide exposure. The origin of somaclonal variation is unknown at this time, however it is widely acknowledged as a probable source of genetic variety.

2.5. Mutagenesis

Somaclonal variations are not always adequate to obtain desirable traits to be isolated from in vitro cell and tissue-based systems. Consequently, in plant cell and tissue cultures, mutagens are commonly utilized to promote genetic variety. In vitro cell and tissue-based systems have great mutation potentials that occur through alterations in karyotype, gross structural rearrangements of chromosomes, transposable elements, point mutations DNA methylation and minor modifications in the structure or location of single or more genes. The culture of plant cell can be used for efficient mutagenesis because the cells can be consistently treated with chemical or physical mutagens.

For mutagen exposure in plant tissue and cell cultures, a number of cell sources can be employed. Physical mutagens and chemical mutagens are used to induce mutations in plants. X-rays and gamma rays are frequently used physical mutagens and diethylsulfate, ethyl methanesulfonate and N-nitro N-methyl urea are frequently used chemical mutagens. In vitro cell and tissue-based systems, chemical mutagens are mostly accepted as better mutagens over the physical mutagens (Wood et al., 1983;

Gavazzi et al., 1987) applied ethyl methane sulphonate to create mutant progenies in tomato in vitro culture. They detected several mutant regenerants having male sterility, seedling lethality, short stature, resistance to *Verticillium*, and change in number of lateral shoots and in leaf shape. Application of mutagens in vitro cell and tissue-based systems, particularly through the use of gamma ray had been speeded up the breeding program to obtain heritable variations (Ahloowalia et al., 2004; Maluszynski et al., 1995). The main limitations to the use of mutagens in tissue culture for crops improvement are the reduction or absence of regenerated plant and undesirable feature changes like reduced fertility and growth. Somaclonal variations occur in rather high frequencies in vitro and have great advantages over conventional application of mutagens. Appropriate culture techniques and effective selection methods can increase the frequency of desirable plant traits (Karp et al., 1989).

2.6. Cell and tissue cultures used for in vitro herbicide resistant plant selection

2.6.1. Callus culture

Mauvais and Chaleff (1984) developed chlorsulfuron-resistant mutants from tobacco leaves generated haploid callus under in vitro conditions. Uneven herbicide application on callus tissue to the selection resistant callus can easily result in a mixture of mutant and normal cells. Consequently, to compensate for the herbicide's uneven exposure, callus is frequently sprayed with the herbicide many times. A single cell can form plant callus, and numerous callus can regenerate the entire plant (Steward et al., 1958; Nagata and Takebe, 1971). To obtain undifferentiated callus, plant shoots, roots, leaves, and anthers can be utilized as explants. Callus cells also undergo somatic embryogenesis, under certain conditions. Somatic embryogenesis is one of the most significant micro-propagation methods that can be utilized as a tool for crop improvements. Callus induction and then plant regeneration are the main steps for utilization of these methods. Consequently, some callus formations are involved in cell dedifferentiation. (Iwase et al., 2011) reported that different types of callus in Arabidopsis thaliana had different gene expression profiles. Callus can be induced artificially in vitro conditions (Gautheret, 1939; Nobécourt, 1939; Hite, 1939) and that the condition of dedifferentiation and differentiation is determined by the balance of cytokinin and auxin

(Skoog and Miller, 1957). Both fundamental research and industry applications have made extensive use of Callus (George and Sherrington, 1984; Bourgaud et al., 2001).

2.6.2. Cell suspension culture

The cell suspension culture is preferred since it offers standardization for cell reproduction and desired cells selection (Shayakhmetov and Asfandiyarova, 1991). Cell number and cell weight in suspension cultures can be measured (Davis et al., 1984). Suspension cultures also offer the measurement of growth rates in different growth stage of cultures (Ray and Still, 1975). After lag phase of the growth little cell growths can be detected. After lag phase, logarithmic increase in cell growth rate can be detected. In suspension cultures growth rate of cells can change constantly from one subculture to another (Street, 1973). Thus, the cultures of cell suspension must be maintained and the culture must be observed in each growth stage to select the suitable culture. Some challenges may arise during cell selection, such as distinguishing between sluggish population adaption and fast expansion of a rare cell variation (Street, 1973).

2.6.3. Plated cell culture

Plant cell culture methods offer a potentially renewable supply of herbicide-resistant cell variations that are difficult to choose in the field. Therefore, plant cell cultures are more advantageous to use and to easily - identify the growing colonies of resistant variants (Thiemann and Barz, 1994). Cell variant isolations from cell cultures are a substantial tool for advancing in plant metabolism, development, and crop improvement in tissue cultures (Conner and Meredith, 1984) indicated that plated cell cultures were the most efficient method for selection of desirable variants compared to suspension and callus cultures. Cells are typically cultivated to the middle or late log phase of their development in semi-solid non-selective media. When the cells are homogeneously exposed to the herbicide, the rare variant cells can be easily seen in the cell population.

2.6.4. Protoplast culture

Different enzyme systems and medium compositions have been used to create plant protoplasts. Plant protoplasts may be isolated from root meristems, shoot, roots and young leaves (Vasil 1976). Protoplasts can be encouraged to regenerate in order to

produce plants immediately, or cell cultures can be used to produce plants later. Leaves and shoot protoplasts are perfect in vitro plant material since they produce genetically identical cell cultures. Csépl'ó et al. (1985) reported that leaf protoplasts of tobacco were used to develop triazine resistance plant. Even though, protoplast cultures allow for selection of mutant variants compared with cells and callus cultures, protoplast cultures are sophisticated and need considerable caution for effective culture (Hickok, 1987). In protoplast culture, plating efficiency is relatively low and specialized techniques are needed to support the feeder cells in the protoplast culture.

2.6.5. Macrospore and microspore cultures

Immature pollen and ovary are mostly used to obtain haploid plants. Both pollen and ovule cultures are very simple techniques. Since it is easy to select pollen and ovule at different developmental stage, adjust medium composition (Collins, 1977). Recessive mutations can be easily detected in haploid plants and dihoploid plants can be obtained by chromosome doubling with colchicine treatment.

Rathore et al. (1993) reported protoplasts of altered rice (*Oryza sativa*) for the generation of herbicide-resistant rice. The bar gene was used with the combination of herbicide Basta. Transmitted bar and gusA genes made rice plant resistant to herbicide Basta. Herbicide Basta was used as a post-emergence on rice plants transformed with the bar gene (Menczel et al., 1986). Confirmed that an important Indica rice cultivar *Oryza sativacv*. IR72 was transformed with the application of direct gene transfer. The transformed rice showed resistance to high dosage level of phosphinothricin.

Herbicide resistance or tolerance is of the most desirable traits in modern biotech crop cultivars. Over the last decade, developed herbicide resistant/tolerant crops to specific herbicides made a great contribution to weed control. Several imidazolinone (IMI) herbicide resistant crops (acetolactate synthase (ALS, EC 2.2.1.6; also known as acetohydroxyacid synthase: AHAS) have been created. IMI resistant crops are known as "IMI cultivars," and their development mainly involves Clearfield technology. In vitro selection is one of the most efficient selection techniques for mutations having herbicide resistant/tolerant. Since the first in-vitro selection for resistance or tolerance to herbicides, numerous examples of success has been achieved for a number of crop

species. Plant cell culture techniques offer mutant selection in large cell populations. In the present study, direct selection in black cumin callus cultures was utilized to generate IMI resistant mutants.

Imazapic, imazapyr, imazamox, imazethapyr, imazaquin, and imazamethabenz are imidazolinone herbicides that suppress weeds by inhibiting the synthase of enzyme acetohydroxyacid (AHAS), commonly another name for it is acetolactate synthase (ALS). In plants, the enzyme AHAS is essential for the synthesis of branched-chain amino acids. Several alternative AHAS genes imparting imidazolinone resistance in plants were discovered by mutagenesis and selection, which were used to develop imidazolinone-tolerant rice (*Oryza sativa* L), maize (*Zea mays* L), wheat (*Triticum aestivum* L), sunflower (*Helianthus annuus* L) and oilseed rape (*Brassica napus* L), and (Tan et al., 2005).

Herbicide resistance is normally passed on to crops by the one of the three systems of action: metabolic detoxification, site of action resistance, or preventing the herbicide from reaching the target (Sherman et al., 2018). Herbicide resistance in a crop might be achieved by genetically modifying one or more of these three pathways.

2.7. Mode of action

Herbicides based on imidazolinone kill weeds by blocking the enzyme acetohydroxyacid synthase (AHAS), also called acetolactate synthase (ALS), which is required for plants to synthesize branched-chain amino acids. These herbicides effectively manage a wide range of grass and broadleaf weeds at low treatment rates, are non-toxic to mammals, and are ecologically acceptable. As a result, imidazolinone herbicides have a number of desirable properties for use in herbicide-resistant crops. In addition, imidazolinone-tolerant plants have been discovered in a number of crops with altered AHAS genes and enzymes. It is now possible to develop imidazolinone-tolerant crops based on the mechanism of resistance at the site of action for these crops. Since the commercial debut of imidazolinone-tolerant maize in 1992, four more imidazolinone-tolerant cultivars have been developed and produced using traditional breeding methods. Furthermore, many imidazolinone-tolerant crops have been registered with four active components, either alone or in combination. Extensive

research and development on this multi-trait and multi-herbicide technology has been possible because to a public-private cooperation (Tan et al., 2005).

2.8. The interaction between AHAS enzyme and IMI herbicides

According to earlier research and the crystal structure of yeast AHAS, the eukaryotic AHAS enzyme may be made up of a catalytic and a regulatory subunit (Hershey et al., 1999; Pang et al., 2002). Most likely, the catalytic component is a homodimer generated by folding two big subunits together (LSU); and each LSU is a monomer of the AHAS polypeptide, which has three similar-sized domains γ, α and β (Pang et al., 2002; Duggleby et al., 2003; Bekkaoui, Schorr et al., 1993). In eukaryotes, the regulatory subunit is thought to be an AHAS small subunit (SSU) (Hershey et al., 1999; Lee and Duggleby, 2002). According to structural modeling of plant AHAS, crystal structure research of yeast AHAS, and data on known AHAS mutations, herbicides that inhibit AHAS bind to the active site identified at the interface of the two LSU monomers in the catalytic subunit of AHAS (Ott et al., 1996; Pang et al., 2002). Herbicide-tolerant mutations can be identified in all three domains, however the protein folds in such a way that the herbicide-binding site is thought to be at the interface between the two monomers, where all relevant amino-acid substitutions may be detected (Duggleby et al., 2003). Some amino acids in the predicted herbicide-binding pocket boosted AHAS resistance to imidazolinone herbicides, whereas others in the pocket increased AHAS sensitivity to imidazolinone herbicides (Ott et al., 1996; Duggleby et al., 2003). Both data suggest that the herbicides interact with the amino acids in the hypothesized herbicide-binding pocket. Different types of herbicides are thought to bind to separate but overlapping locations inside the pocket (Schloss 1990; Ott et al., 1996; Preston and Mallory-Smith 2001; Pang et al., 2002). The binding pocket is expected to be near the entrance point for the AHAS enzyme's substrate, according to molecular modeling of the AHAS–imidazolinone interaction (Ott et al., 1996). The herbicide docking model's findings, along with AHAS crystal structure studies, show that each imidazolinones and sulfonylureas may bind in the access channel to the substrate, preventing attaching the substrate with AHAS (Ott et al., 1996; Duggleby et al., 2003).

The main composition of LSU AHAS is made up of around 670 amino acids, which varies according on the species (Mazur et al., 1987; Bernasconi et al., 1995; Ott et al., 1996; Pang et al., 2002). Many researchers refer to these two places as regions or domains (A and B) because amino-acid changes in domains and result in the bulk of imidazolinone-tolerant mutations (Wright et al., 1998; Tranel and Wright, 2002). AHAS gene mutations that give resistance to AHAS inhibitory herbicides in plants have been examined by several authors (Tranel and Wright, 2002; Preston and Mallory-Smith, 2001; Gressel, 2002). Resistance to AHAS inhibitors is most often conferred by mutations in the AHAS LSU at several locations Trp574, Ala205, Ala122, Ser653 and Pro197. The Ser653 mutation provides resistance to imidazolinones but not to other AHAS inhibitors, which is a desirable trait for the Imidazolinone-tolerant crops (Lee et al., 1999; Tranel and Wright 2002; Tranel 2006). The Trp574 mutation has been exploited to generate imidazolinone-tolerant crops because of typically it is cross-tolerant to multiple types of herbicides inhibiting AHAS (Bernasconi et al., 1995; Wright and Tranel, 2002). Imidazolinone tolerance is acceptable in Ala122 and Ala205 mutations, making them a suitable candidate for developing imidazolinone-tolerant crops. The mutation at Pro197, on the other hand, shows no or limited tolerance to imidazolinones but high tolerance to sulfonylureas (Bright et al., 1992; Yu et al., 2003). Currently, the significant number of imidazolinone-tolerant crops on the market are derived from the Ser653, Trp574, or Ala205 mutations (Bruniard 2001; Clemente et al., 2002). All commercially available imidazolinone-tolerant crops were produced using traditional plant breeding approaches, such as selection or mutagenesis, and are thus non-transgenic (Mangels et al., 1991; Endo and Toki, 2013). A target-site variation in the AHAS enzyme decreases its susceptibility to imidazolinone herbicide., resulting in imidazolinone tolerance. Due to the fact that other herbicides tolerance mechanisms (metabolism, decreased absorption, and translocation to the catalytic site) may contribute to imidazolinone tolerance, these intrinsic plant features have not been investigated in the creation of imidazolinone-tolerant plants.

2.9. Imidazolinone tolerant plants that have been commercialized

2.9.1. Imidazolinone-tolerant oilseed rape

Table 2.3. Examples of IMI-resistant crops developed via classical breeding.

Crops		Reference
Scientific name	**Common name**	
Helianthus annuus L.	Sunflower	(Monks et al., 1996)
Brassica napus L.	Oilseed rape	(Berthelot and Carpentier 2003)
Oryza sativa L.	Rice	(Croughan 2003)
Triticum aestivum L.	Wheat	(Hu et al., 2015)
Zea mays L.	Maize	(Alonso et al., 1998)

Canola microspores were collected, mutagenized using ethyl nitrosourea, and grown into embryos, haploid plantlets, and then doubled using colchicine (Swanson et al., 1989). There are five double-haploids escaped imazethapyr soil remediation, and both of the lines showed increased herbicide resistance. P2 and P1 also known as PM2 and PM1, were the two most tolerant mutants (Swanson et al., 1989; Dale L Shaner et al., 2018). PM1 and PM2 mutations were used to generate all of imidazolinone tolerant oilseed rape cultivars, which were initially sold as Smart canola in 1995.

Brassica napus is thought to be the result of an interspecific hybrid between the C genome donor *Brassica oleracea* L and A genome donor *Brassica campestris* L. (Rutledge et al., 1991; Poehlman and Sleper, 1995). In oilseed rape, five AHAS loci have been identified. The A genome produces AHAS4, AHAS2, and AHAS43, whereas the C genome produces AHAS5 and AHAS1 (Rutledge et al., 1991).

2.9.2. Imidazolinone-tolerant rice

Ethyl methane sulfonate (EMS) was used to mutagenize rice seeds from the AS3510 variety. Imazethapyr was sprayed on M2 plants. A solitary surviving plant was found, and the rice plant's offspring were resistant to numerous AHAS-inhibiting herbicides (Endo and Toki 2013) 93AS3510 was the name given to this mutant line, and two rice variants resistant to imidazolinone, CL141 and CL121, they were launched in the United States with this tolerance feature (2001).. by using EMS a total of 54 and 55 seeds of the rice cultivar Cypress were mutagenized (Clemente et al., 2002). Imazapyr

or imazapic were applied to the leaves of M2 plants. Twelve plants made it through the treatment and were found to be tolerable. PWC29, CMC37, PWC16, CMC23, WDC31, WDC38, and WDC33, the seven most tolerant lines, were chosen for further study (Clemente et al., 2002). Following that, two types of rice imidazolinone tolerant, XL8 and CL161, were created from the source's mutations and released in 2003 (Geal et al., 2003).

2.9.3. Wheat resistant to imidazolinone

Winter wheat seeds were mutagenized with sodium azide and an imazethapyr seed treatment was followed by imazethapyr pre-emergence application to screen the M2 seeds. Fidel selection (FS) FS1, FS2, FS3, and FS4 were chosen as the 4 tolerant plants (Newhouse et al., 1992). Following that, these field selections of imazethapyr tolerant were utilized as donors for trait in the development for varieties of the imidazolinone-tolerant wheat, which were initially commercialized in 2001. Seed mutagenesis applications also led to the discovery of more wheat mutants (Pozniak and Hucl, 2004). Imazamox was sprayed on spring wheat M2 plants. For additional genetic research, six lines with imazamox tolerance levels ranging from moderate to high were selected.

2.10. Plant tissue culture

The development of plant tissue and cell culture has made great progress during the previous four decades. Three main technologies make up the majority of what are referred to as plant in vitro technologies or tissue culture. Plant tissue growing on gel-solidified nutrition medium is the most frequent method. This technique is utilized in the most crucial operations; such as plant regeneration from cultured cells. Plant cell culture in liquid suspension was created soon after, and it has shown to be a very successful method for somatic embryogenesis plant regeneration. The demand for virus-free plants prompted the development of the meristem culture method. The method is currently being utilized to create virus-free agricultural plants in a variety of species. Another significant technique is the production of haploid and homozygous plants using anthers and microspores. The development of plant protoplast and cell fusion technologies for the production of novel plant hybrids is part of plant tissue culture. The third component of the progression is the marriage of tissue culture and molecular genetics, which has evolved into the fast rising field of biotechnology (Gamborg, 2002).

2.11. The formation of Callus

Stem cells from animal tissues are often recognized to grow into tissue cells that are finally terminated. However, plant tissue cells that have been differentiated are hypothesized to be able to mending damaged tissues and de-differentiating, if not the entire plant; they are also thought to be capable of forming totipotent callus cells (Chen et al., 2000; Burris et al., 2009)

Plant cells do not re-differentiate, according to a more recent theory, but calluses are produced from pre-existing stem cells (Sugimoto et al., 2011; Wang et al., 2011). The molecular mechanisms that underpin stem cell differentiation and/or somatic plant cell differentiation–dedifferentiation are not well understood. Stem cell-related genes are required for dedifferentiation processes. Epigenetic mechanisms including histone modification and DNA methylation, as well as transcription factors, regulate their expression (Jiang et al., 2015).

The dynamics of the geographical and temporal distribution of hormonal and developmental meristem regulators were investigated using microscopic live-cell imaging. WUSCHEL (WUS) expression, auxin-mediated primordia placement, and other growth determinants are all influenced by microtubules, transcription factor networks, and cytokinin pathways (Sijacic and Liu, 2010).

2.12. The somaclonal variation

Somaclonal variation is a type of variation that develops in tissue cultures and cells (Scowcroft and Larkin, 1981). The somaclonal variation is now widely used to describe all types of tissue culture-derived variations (Bajaj 1990). However, protoplast, anther, and meristem culture differences are often described using terminology like mericlonal, gametoclonal and protoclonal variation (Chen et al., 1998 Karp, 1994).

Some scientists added a requirement that somaclonal variation be heritable during the reproductive cycle to the concept. Due to complicated sexual incompatibility, seedlessness, polyploidy, or lengthy generation cycles, it is not always easy to establish heredity. As a result, it may be difficult, if not impossible, to explain the heritable character of somaclonal variation in these plants (Skirvin et al., 1994). Braun (1959)

was the first to notice and record somaclonal variation, and he was the first to report it. It has been and continues to be one of the most serious issues with many tissue grown plants. Plant cell production in vitro and regeneration into full plants is an asexual process that needs just mitotic cell division and should produce no variation in principle. The presumption is that genetically homogenous plants will reproduce clonally (Jain et al., 1998).

As a result, the development of random spontaneous variety and uncontrolled in the cultural process is frequently unanticipated and undesirable (Angela Karp, 1994).

Despite these negative repercussions, its utility in crop improvement via the generation of new varieties has been well demonstrated (Predieri, 2001; Mehta and Angra, 2000) To alter the genetics of polygenic crops, induced somaclonal variation can be used (Jain 2001). It may also be used in plant breeding to create new cultivars with better disease resistance, quality, and yield (Angela Karp, 1995; Emaldi et al., 2004).

2.13. The relationship between somaclonal variation and mutation induction

2.13.1. Heritable alterations in the DNA

Mutations are changes in gene sequence that are not induced by genetic segregation or recombination (van Belkum et al., 1998). Specific treatments comprising chemical and physical mutagens, as well as tissue culture, can be used to generate genetic variation in mutants (Roux, 2004; Predieri, 2001). Surprisingly, the frequency of manifestation of dominance has been shown to be greater in somaclonal variation than in other types of mutation (Yang et al., 2010; Jain et al., 1998). The function of tissue culture in generating mutations other than those caused by physical or chemical mutagens has been debated for a long time (Jain et al., 1988) remained a mystery. Although much study has been done on induced somaclonal variation and mutation separately, additional effort is needed to fully harness their combined beneficial benefits. There is a growing amount of evidence that favorable impacts may be created by combining mutagenesis with tissue culture in ornamental and agricultural plants (Bairu et al., 2011). Many research have demonstrated that in vitro culture may be utilized to develop plants with increased genetic diversity and mutants as a source of new commercial cultivars, either alone or in conjunction with mutagenesis caused by physicochemical

and biological agents (Rasheed 2003; Orbović et al., 2008). Organ cultures, calli, protoplasts, and somatic embryogenesis plants can show phenotypic and DNA variations, and in vitro culture conditions can be mutagenic (Orbović et al., 2008; Bouharmont, 1994). Mutagens can be utilized on undifferentiated tissues and organs that do not have pre-existing axillary buds prior to regeneration or at various stages of adventitious meristem differentiation (Predieri, 2001). Consequently, some of the resulting somaclones may have favorable characteristics that are heritable by progenies and stay stable over time (Mandal et al., 2001). Induction of somaclonal variation has also been helpful in discovering possible novel varieties in a variety of crops, including potato and millet. Most significantly, tissue culture improves the efficacy of mutagenesis therapies for inducing variety, enables for huge populations to be handled, and allows for fast cloning of chosen variations under strict phytosanitary conditions (Bairu et al., 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1. Method of seeds sterilization

The black cumin (*Nigella sativa* L.) seeds were obtained from Erciyes University, Agriculture Faculty, Department of Agricultural Biotechnology Kayseri, Turkey. The black cumin cultivar Çameli was used as plant material. Seeds were washed under running top water in a cheese cloth for 30 minutes. The seeds were sterilized by soaking for 5 minutes in 50 percent regular bleach containing sodium hypochloride (NaOCl) (3%) and then rinsing three times in sterile distilled water.

3.2. Seeds germination and growth conditions

Seeds were then germinated on Petri dishes lined with filter paper (sterilized in the autoclave at 121°C, pressure 104 kg/cm² for 15 min.) soaked with 5 ml of sterile distilled water in growth chamber (Figure 3.1). Ten seeds cultured in each Petri dish. The Petri dishes were replaced in the growth chamber at 26 +1 ºC with a photoperiod of 16 hrs. light. The Petri dishes were checked every day about contamination rate and about seed germination. The infected Petri dishes were eliminated. After 4 weeks, uniform germinated seedlings were chosen for callus induction in the laminar flow. The cotyledon, cotyledon nodes and hypocotyl of the explants were separated aseptically under sterile conditions.

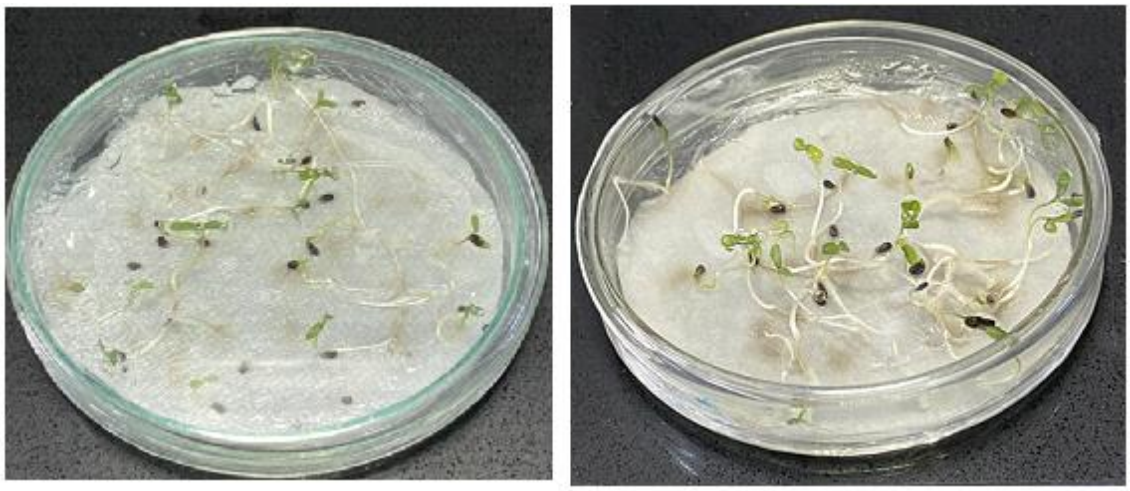

Figure 3.1. Aseptically germinated black cumin seeds

3.3. Method of callus establishing and culture media content

The callus was established from hypocotyl, cotyledon and cotyledon node explants of three weeks old plants germinated in vitro. The culture media consisted of inorganic salts, organic compounds, vitamins, gelling agent. The culture components were dissolved in distilled water. Auxins and cytokinins were dissolved in 0.5N ethanol and dilute 0.1N HCl, respectively. For callus initiation, MS basal medium (Murashige and Skoog, 1962; Table 1) was used. Sucrose was added as a carbohydrate source. The callus was maintained in MS mineral salt and vitamins, 3% sucrose and 0.7% agar (Type A, Sigma) and sub-cultured every three weeks were used for plant growth medium. The pH of the callus media was adjusted to 5.8 by using with 0.5 N NaOH or 0.1 N HCl prior to autoclaving. The culture medium was steam sterilized by autoclaving at 15 psi pressure at 121°C for 15 minutes. The medium was poured in the culture vessels.

Table 3.1. Composition of Murashige and skoog (MS) medium.

Ingredients	Amounts (mg/L)
1- Macronutrients	
NH4NO3	1650
KNO3	1900
CaCl2. H2O	440
MgSO4 7H2O	370
KH2PO4	170
2- Micronutrients	
CuSO4.5H2O	0.025
Kl	0.83
H3BO3	6.2
MnSO4.4H2O	22.3
ZnSO4.7H2O	8.6
Na2MoO4.2H2O	0.25
CoCl2.6H2O	0.025
FeSO4.7H2O	27.8
Na2EDTA.2H2O	37.3
3- vitamins	
Inisitol	100
Thiamine-HCl	0.1
Nicotinic Acid	0.5
Pyridoxine-HCl	0.5
Glycine	2

The explants were cultivated at a temperature of 25 ± 1 ºC with a photoperiod of 16 hours of light. Every three weeks, the subcultures were carried out in the same medium and under the same growth circumstances.

The explant cultures were housed in a growth chamber at a constant temperature of 25±2°C with 14h/d illumination of 30 to 40 mol m-2s^{-1} Spectral Flux Photons (SFP) and a relative humidity of 60-70 percent (RH). Cool white fluorescent bulbs supplied the illumination. The temperature was kept at a comfortable level by the air conditioning system.

On hypocotyl, cotyledon node, and cotyledon explants, several hormone combinations of NAA (1 and 2 mg/L) and BA (1, 1,5, 2, 5, and 3 mg/L) were employed for callus formation. Following the plant regeneration experiment, the calluses were assessed based on the shoots and lengths of the shoots, and the shoots were planted separately into various rooting conditions.

After determination of the optimum concentration of plant growth regulator (2,4-D and kin) and the best plant part on the experiments which were done, all following media were prepared with 1.5 mg / L kin and 1 mg /L 2,4 –D to keep callus growing, and the leaf chosen to be best explant which gives good yield callus induction and also give green callus color above other explants (stem and root).

The filterized Imazamox (IMI) was mixed with MS medium culture at a temperature of 40 – 45°C under aseptic condition. The proliferated calli were placed into MS medium supplemented with 3.0 mg/l 2.4 D + 1.0 mg/l Kin and 0.00, 0.25, 0.50, 1.00, 2.00, 4.00µM concentration of IMI herbicides. In three weeks, visual assessments were evaluated and recorded on the different treatments of IMI.

For induction of somatic embryogenesis, the proliferated calli were transferred to MS media with a PGR-free medium for further stimulation of somatic embryogenesis. The percentage of plant regeneration was calculated based on the number of regenerated explants/the number of induced calli with embryogenic potential in each petri dish. Both regeneration and somatic embryogenesis experimental cultures were stored in the growth chamber at 25±1°C under a 16/8 h light photoperiod.

The experimental design was a completely randomized design with 4 replications. Data was expressed as mean values per culture, were subjected to analysis of variance using the GLM procedure in SAS v. 9.4 (SAS Institute Inc., Cary, NC) and the means were compared by the LSD test (($P< 0.01$).

CHAPTER 4

RESULTS

4.1. Callus production

Callus was initiated from cotyledon and hypocotyl explants, each grown under light conditions (Figure 4.1-2). In the current thesis study, the approximate size of the initial callus population was determined by visual evaluation (as best I could) of the percent of callus on the media culture. The callus cultures employed in this thesis project were derived from cotyledon and hypocotyl explant that had been produced by aseptically germinated seeds of black cumin. Callus production rate of cotyledon and hypocotyl explants was given in Table 4.1. When treated with 3.0 mg/L 2.4 D + 1.0 mg/L Kinetin, the callus production rates of cotyledon and hypocotyl was 91.2 and 86.2 %, respectively. The highest callus production rate was obtained from cotyledon explant.

Table 4.1. Percentage of callus production of cotyledon and hypocotyl explants after fifteen days of cultivation.

Treatments	Cotyledon callus rate (%)	Hypocotyl callus rate (%)
3.0 mg/l 2.4 D + 1.0 mg/l Kin	91.2 a	86.2 b
LSD 0.05	1.25	

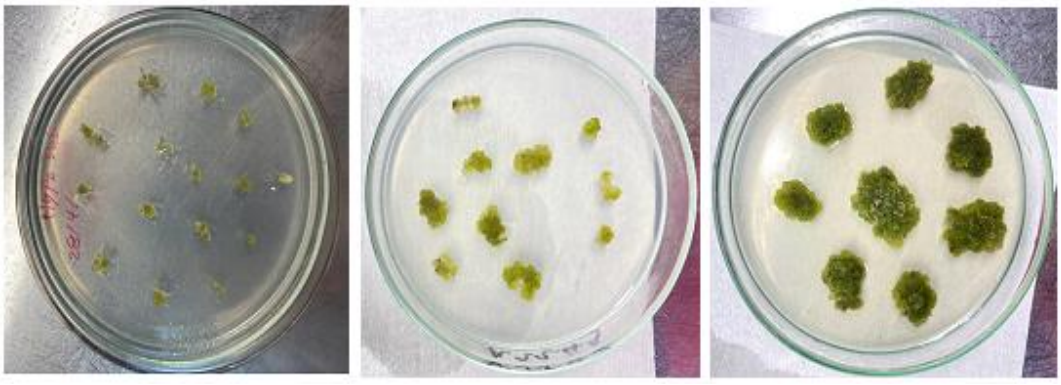

Figure 4.1. Callus production from the cotyledon of black cumin seedlings

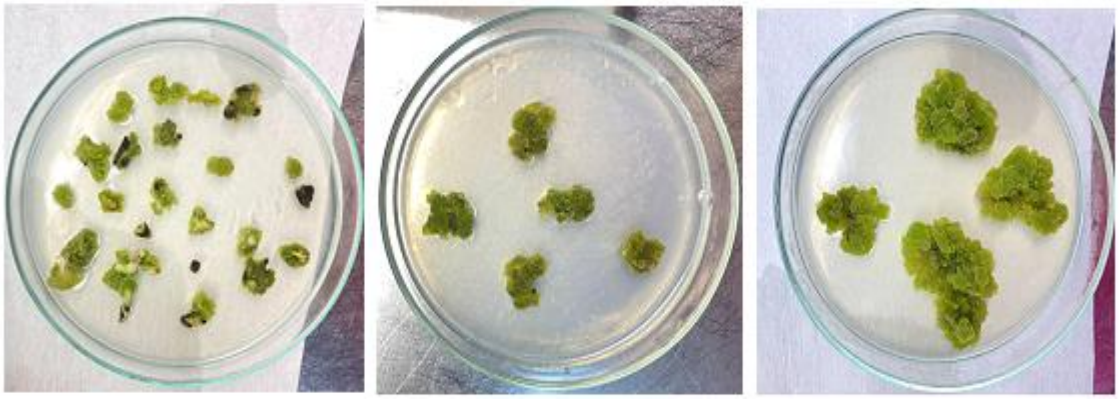

Figure 4.2. Callus production from the hypocotyl of black cumin seedlings

Black cumin callus obtained from cotyledon and hypocotyl explants were transferred on the fresh agar based medium after 6 weeks of incubation. The transferred black cumin callus on the fresh agar based medium exhibited a characteristic cell growth pattern during a four-week period of the cultivation (Figure 4.3). After three weeks of cultivation, rapid callus growth was observed.

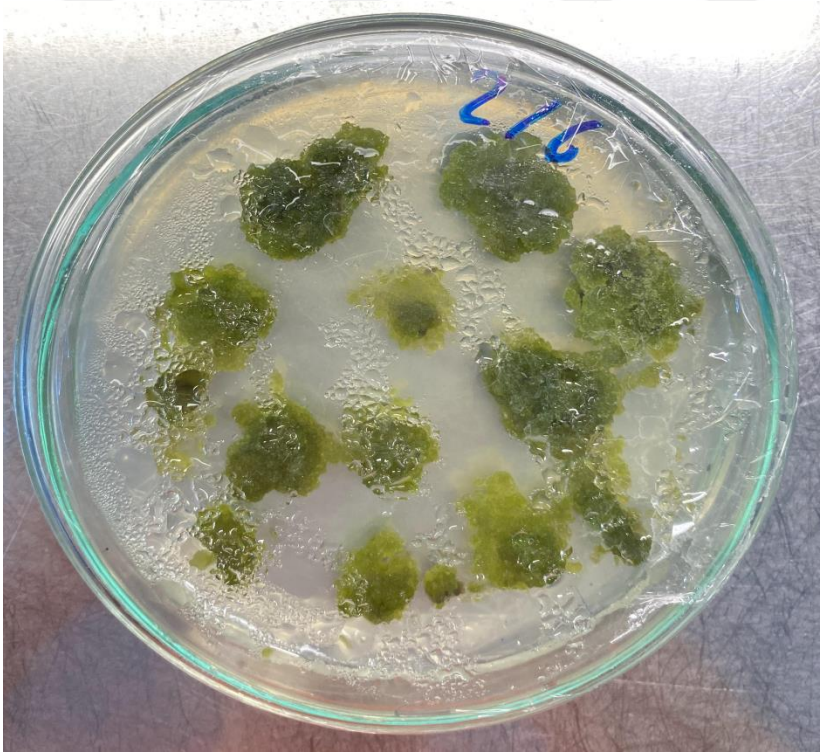

Figure 4.3. Black cumin callus grown on agar based medium for a four-week period.

4.2. Imidazolinone (IMI) treatment

The applied IMI doses were significantly reduced callus growth rate. The growth rate of IMI treated callus varied between 94.00 and 64.35 % (Table 4.2). The highest callus growth rate was obtained from no IMI treated control group and the highest was obtained from 4.00 m/L IMI treatment. Color of IMI treated callus tissue varied from dark green to yellowish green. The IMI herbicide reduced the chlorophyll content of the callus tissue. When somatic embryo production was considered, the highest somatic embryo was obtained from no IMI treated group with11.66 embryo/callus, while the lowest was obtained from 1.00, 2.00 and 4.00 ml/L IMI treatments, respectively.

Several features of the growth of black cumin cotyledon and hypocotyl explants in culture made it difficult to observe and determine the frequency of occurred mutation from IMI sensitivity to IMI resistance. Growth of non-mutant cells in the callus was slightly inhibited by 400 mM IMI application (Table 4.2).

Table 4.2. Callus growth rate, callus color and shoot number after thirty days of IMI treatment.

IMI treatment (μM)	Callus growth rate (%)	Callus color	Somatic embryo number	Shoot number
0.00	94.00 a	Dark green	11.66	0
0.25	91.30 ab	Light green	4.34	0
0.50	87.33 bc	Light green	2.00	0
1.00	84.64 c	Yellowish green	0.00	0
2.00	80.00 d	Yellowish green	0.00	0
4.00	64.35 e	Yellowish green	0.00	0
LSD 0.05	4.37		1.39	

Spontaneous mutants appeared at quite low frequency. Therefore, it was visually very difficult to detect them.

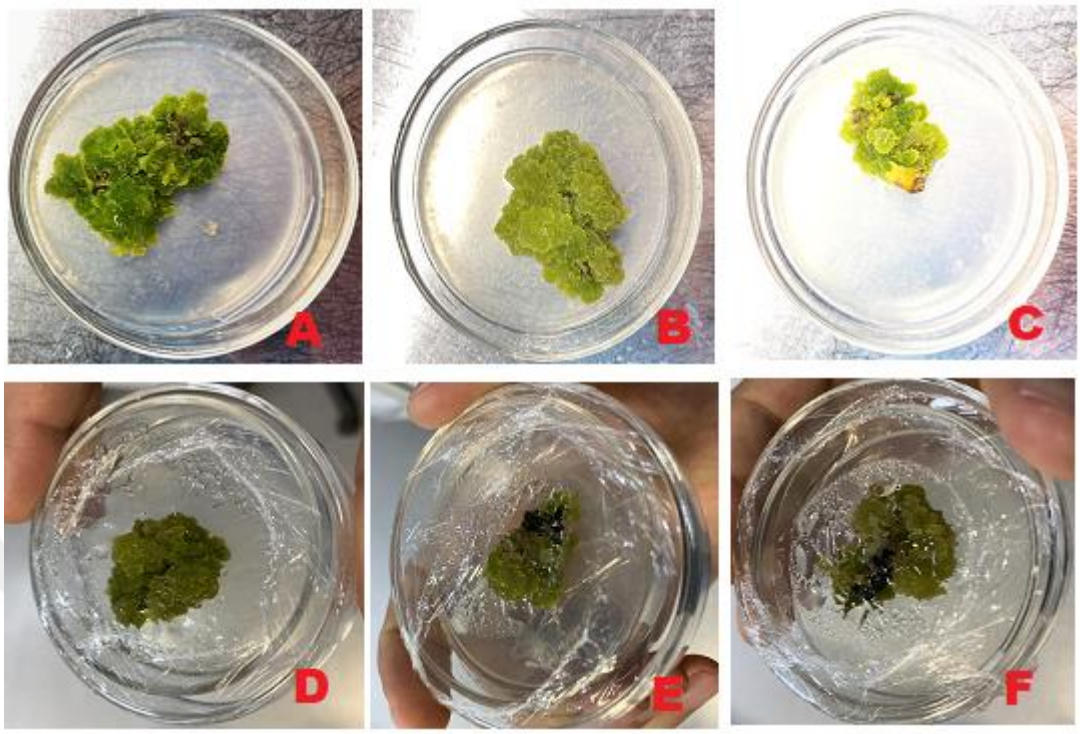

Figure 4.4. IMI treated black cumin callus after thirty days of application. A: 0.00 µM, 0.25 µM, 0.50 µM, 1.00 µM, 2.00 µM and 4 µM IMI application.

The color of callus after application of different IMI doses was varied between dark green to yellowish green. No IMI applied control group had the dark green color, while 2 and 4 µM IMI applied groups had yellowish green color (Table 4. 2).

When somatic embryo number was in consideration, embryo number varied between 0 and 1.66 callus/somatic embryo. The highest somatic embryo as obtained from no herbcde applied control group and the lowest was obtained from 2 and 4 mM IMI herbicide applied groups (Table 4. 2). There was no shoot number observed in all treatment groups.

CHAPTER 5

DISCUSSION

The main purpose of the current study was to develop IMI herbicide resistant black cumin genotypes. The IMI group herbicides are very effective to control broad range of weed species in most of the economically important crops. When IMI herbicides translocate into the site of action, they characteristically inhibit acetolactate synthase, and cause meristematic cells death that results in plant death (Little and Shaner, 1991).

There are two approaches, conventional plant breeding and biotechnological approaches, to develop herbicide tolerant crops. Conventional plant breeding approach has some limitations since naturally herbicide resistant plants are found more often among weed species than in crop species. Also, conventional plant breeding takes a long time to develop herbicide resistant crops. However, biotechnological approaches such as genetic transformation of resistance gene and in vitro cell cultures are much faster than conventional plant breeding approach. The utilization of callus culture offers to develop variant cell lines that have been tolerant or resistant to herbicides. It is important to note that the in vitro method requires a long period of cell or tissue culture and needs immediate plant regeneration from tolerant/resistant cell lines. Currently, numerous herbicide-resistant crops have been developed and commercial in the World. White clover (*Trifolium repens*) cell lines tolerant to several phenoxy herbicides were reported (Oswald et al., 1977), and cell lines of tobacco (*Nicotiana tabacum*) resistant to amitrole (3-amino-1,2,4-triazole) were also isolated (Barg, and Umiel, 1977; Swartzberg et al. 1985).

Imidazolinone resistance crops were the first herbicide-resistant crops developed through non-classical methods (Greaves et al., 1993). Imidazolinone herbicides producer company American Cyanamid introduced a global brand name covering imidazolinone-resistant and imidazolinone-tolerant crop varieties from all companies.

All imidazolinone-resistant and imidazolinone-tolerant crop cultivars have been recognized by the “CLEARFIELD” name in 2000.

The imidazolinone herbicides were developed in the 1980s (Los, 1987; Shaner, 1991). Their mode of action is similar to sulfonylureas. Both imidazolinone and sulfonylurea herbicide groups inhibit ALS (Chaleff and Mauvais, 1984; Shaner, et al., 1984) although both herbicides are chemically different (Stidham 1991). Due to their effectiveness and less toxicity to human health and environment, imidazolinone herbicides are very popular (Mazur et al., 1987; Bernasconi et al., 1995). The current study was conducted to isolate IMI herbicides resistant cells from cotyledon and hypocotyl explant cultures which were known to have a specific metabolic target. It was hoped to generate mutant material cells carrying the resistance gene could be regenerated and used in black cumin breeding programs. Callus cultures of black cumin were chosen since it has been widely used to generate mutant herbicide resistant variants (Chaleff and Mauvais, 1984; George and Sherrington, 1984; Bourgaud et al., 2001).

The profitability of the imidazolinone-resistant black cumin production compared with conventional black cumin production will depend on the seed yield increase and cost of seed, herbicide and application expenses. To the best of my knowledge, there has been no record on development of IMI resistant black cumin. This thesis project was the first attempt to develop IMI resistant black cumin genotype.

IMI resistant callus cultures were recovered from IMI herbicide applied callus. The failure to obtain IMI resistant regenerants could be attributed to inhibition of cell divisions. Although many herbicide resistant cell lines have been isolated, there have been a few herbicide resistant regenerant plants reported from herbicide resistant cell lines (Carlson, 1973; Maliga, 1976; Hamaker, et al., 1963). Ray (1984) and Shaner et al (1984) reported that the inhibition of cell divisions caused to a block in growth by IMI herbicides and sulfonylurea herbicides. In order to obtain IMI resistant regenerants, the IMI resistant callus must be transferred to IMI free MS basal medium supplemented with appropriate hormone combination and vitamins.

Similar results were reported by Anderson and Georgson (1986) and Shaner et al., (1984) who isolated corn mutant lines resistant to IMI herbicides and they showed that the resistance was resulted from ALS molecule alterations. The existence of imazamox tolerant cell lines in cotyledon and hypocotyl derived calli supported the idea that callus culture was ideal to develop herbicide tolerant black cumin genotypes.

4.1. Conclusion

In this study, callus tissue was obtained from cotyledon and hypocotyl explants of black cumin treated with a combination of 2,4-D and kinetin, and Imazomax herbicide resistant/tolerant cell variants were successfully obtained from imazomax treated calli. Imazomax treatment negatively affected growth rate of callus cultures and plant regeneration. No plant regenerations were accomplished from resistant/tolerant callus cultures. All attempts to regenerate plants from tolerant black cumin callus culture were not successful. The inhibition of plant regeneration from IMI treated callus was attributed to the inhibition of cell division. Therefore, more advanced studies are needed to achieve plant regeneration from IMI treated black cumin callus. Developing imazomax herbicide resistance/tolerance callus offered a promising approach that explores the applicability of callus culture to develop herbicide tolerant black cumin genotypes.

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