ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

Ph.D. THESIS

Abdullatief Mohammed ABDURRUHMAN MOHAMMED

COMPARISON OF DIFFERENT ASSAY TECHNIQUES USED FOR DETECTING ACETYL-CoA CARBOXYLASE (ACCase) AND ACETOLACTATE SYNTHASE (ALS)-INHIBITORS HERBICIDE RESISTANCE IN STERILE WILD OAT (*Avena sterilis* L.)

DEPARTMENT OF PLANT PROTECTION

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We certify that the thesis titled above was reviewed and approved for the award of degree of the Doctor Philosophy by the board of jury on 16/07/2019.

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ABSTRACT

Ph.D. THESIS

COMPARISON OF DIFFERENT ASSAY TECHNIQUES USED FOR DETECTING ACETYL-CoA CARBOXYLASE (ACCase) AND ACETOLACTATE SYNTHASE (ALS)-INHIBITORS HERBICIDE RESISTANCE IN STERILE WILD OAT (*Avena sterilis* L.)

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Intensive use of herbicides has resulted in the occurrence of herbicide resistance. Recently, herbicide resistance has become a global issue threatening agricultural food production. ACCase and ALS-inhibitor-resistant weeds constitute more than one-third of all the reported resistance cases worldwide becoming the main constraint to wheat production in Mediterranean environments. However, different techniques have been devised to detect herbicide resistance in weeds.

This study was aimed to compare the reliability and validity of quick tests [filter paper and agar-based (seed and seedling)], greenhouse (whole-plant pot) and molecular tests for detecting the resistance to acetyl-CoA carboxylase **(**ACCase) and acetolactate synthase (ALS)-inhibiting herbicides in sterile wild oat (*Avena sterilis* L.) in Adana, Turkey. In the molecular test, ACCase genes of the resistance and susceptible populations were sequenced and compared.

As a result, greenhouse tests confirmed that the resistant population of *A. sterilis* has a high-level of resistance to clodinafop-propargyl (11.97 fold) and pyroxsulam (42.88 fold); and low resistance level to pinoxaden (2.1 fold) and mesosulfuron-methyl+iodosulfuron-methyl-sodium (1.08 fold). The levels of resistance obtained from quick tests were less than 5-folds in all herbicides. Six mutation points "Serine (Ser), Glycine (Gly), Threonine (Thr), Valine (Val), Glutamine (Gln) and Phenylalanine (Phe)" were detected in the ACCase gene.

In conclusion, the agar-based tests provided feasible, accurate, rapid and cost-effective herbicide resistance confirmations which need less space than a greenhouse and filter paper tests. The molecular test confirmed the target-site resistance in the resistant population. The study reports these mutations for the first time in *A. sterilis* in Turkey.

Keywords: ACCase herbicides, ALS herbicides, *Avena sterilis* L., resistance, quick test.

ÖZ

DOKTORA TEZİ

ASETİL-CoA KARBOKSILAZ (ACCase) VE ASETOLAKTAT SENTETAZ (ALS) ENZİMİ İNHIBİTÖRÜ HERBİSİTLERE KARŞI KISIR YABANİ YULAF (*Avena sterilis* L.) DİRENCİNİN BELİRLENMESİNDE FARKLI YÖNTEMLERİN KARŞILAŞTIRILMASI

Abdullatief Mohammed ABDURRUHMAN MOHAMMED

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Herbisit direnci, herbisitlerin yoğun bir şekilde kullanımından kaynaklanmış olup, son yıllarda tarım ve gıda güvencesini tehdit eden küresel bir sorun haline gelmiştir. ACCase ve ALS inhibitörlerine dirençli olan yabancı otlar, dünya genelinde bildirilen tüm direnç vakalarının üçte birinden fazlasını oluşturur ve Akdeniz çevrelerde buğday üretiminin ana kısıtlayıcısı olmaktadır. Ancak, yabancı otlardaki herbisit direncini saptamak için farklı yaklaşımlar geliştirilmiştir.

Bu çalışma, Adana ilinde yetişen Kısır Yabani Yulaf (*Avena. sterilis*)'taki Asetil CoA Karboksilaz (ACCase) ve asetolaktat sentetaz (ALS) inhibe eden herbisitlere karşı direnci saptamak amacıyla kullanılan hızlı testler [flitre kağdı ve agar bazlı (tohum ve fide)], sera test ve moleküler testlerinin güvenilirliği ve geçerliliği karşılaştırılmasını amaçlanmıştır. Moleküler testlerde, dirençli ve hassas popülasyonlarının ACCase genleri dizilmiş ve karşılaştırılmıştır.

Yapılan çalışmada, sera testleri, dirençli *A. sterilis* popülasyonunun clodinafop-propargyl (11.97 kat) ve pyroxsulam (42.88 kat)'a karşı yüksek bir direnç seviyesine; ve pinoxaden (2.1 kat) ve mesosulfuron-methyl iodosulfuron-methyl-sodium (1.08 kat)'e karşı düşük bir direnç seviyesine sahip olduğunu doğrulamıştır. Hızlı testlerden elde edilen direnç seviyelerinin tüm herbisitlerde 5 kattan az olduğu bulunmuştur. ACCase geninde altı mutasyon noktası "Serin (Ser), Glisin (Gly), Treonin (Thr), Valin (Val), Glutamin (Gln) ve Fenilalanin (Phe)"" tespit edilmiştir.

Sonuç olarak, agar bazlı testler, doğru, hızlı ve uygun maliyetli herbisit direnci onayları sağlayıp, sera ve flitre kağdı testlerine göre daha az alana ihtiyaç duymaktadır. Moleküler testi, dirençli popülasyondaki hedef bölge direncini doğrulamıştır. Çalışma, bu mutasyonları Türkiye'de ilk kez *A. sterilis*'te rapor etmektedir.

Anahtar Kelimeler: ACCase herbisitler, ALS herbisitler, *Avena sterilis* L., Direnç, hızlı test.

EXTENDED ABSTRACT

Weeds control in the arable area is an essential key step to increase crop production. In the last several years, herbicides have become the most effective tool for weed control. However, repeated and intensive use of herbicide, as well as the limited number of modes of action (MoA) obtainable, has resulted in the appearance of herbicide resistance in weeds. At the beginning of 2019, 500 unique, herbicide-resistant biotypes have been defined worldwide. Today, approximately 45 weed biotypes are resistant to ACCase (acetyl-CoA carboxylase)-inhibiting herbicides and 160 weed biotypes are resistant to ALS (acetolactate synthase)-inhibiting herbicides. Sustainable strategies for weed management using herbicide depend on accurate resistance detection test that permits optimization of treatment solutions that will lead to herbicide longevity.

In this study, we aimed to compare the validity and reliability of different herbicide resistance detection tests [agar-based (seed and seedling), quick (filter-paper based), greenhouse, and molecular-based tests] in sterile wild oat (*Avena sterilis* L.) against ACCase (clodinafop-propargyl+cloquintocet-mexyl and pinoxaden+cloquintocet-mexyl) and ALS (mesosulfuron-methyl+iodosulfuron-methyl-sodium and pyroxsulam+cloquintocet-mexyl)-inhibiting herbicides.

In summer 2016, seeds of resistant (R) *A. sterilis* population were collected from previously known resistance wheat field from Adana province, susceptible (S) seed population was collected from the untreated area. Then the seed was kept in a paper bag and brought to the laboratory, cleaned and stored at 4^{o}C for 60 days in order to break seed dormancy.

Seed germination test was done in Petri-dishes using plant agar (0.9% *w/v*), melted in the microwave and cooled around 35-40^{o}C before herbicide solution was added. The concentrations of 0, 1.2, 2.4, 4.8, 9.6, 19.2 and 38.4 µM for clodinafop-propargyl+cloquintocet mexyl; 0, 0.225, 0.45, 0.9, 1.8, 3.6 and 7.2 µM for pinoxaden+cloquintocet -mexyl; 0, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 µM for

mesosulfuron-methyl+iodosulfuron-mexyl; and 0, 0.37, 0.75, 1.5, 3, 6 and 12 µM for pyroxsulam+cloquintocet-mexyl were used. 20 ml of agar medium was powered into 9-cm diameter glass Petri-dish; ten seeds of R and S populations were placed in petri-dish, covered with the lid and placed in the refrigerator at 10^{o}C for 15 days. The test was arranged in a complete randomized design (CRD) with six replicates of each concentration and the experiment was repeated twice. The result of the test showed that the seed was germinated above 95% in the control of R and S population. More than 20% germination in both populations was founded at the higher concentration of clodinafop-propargyl, mesosulfuron-methyl, and pyroxsulam, whereas less than 10% was germinated in R and S populations treated with pinoxaden. Based on the hypocotyl and radicle lengths, ED_{50} values were calculated, resistance indices (RI's) of hypocotyl were 3, 3, 2.10 and 1.58-fold; RI's of radicle were 1.99, 5.24, 2.67 and 3.32-fold for clodinafop-propargyl, pinoxaden, mesosulfuron-methyl, and pyroxsulam, respectively.

The agar-based seedling test was done in 12 x12 cm diameter plastic Petri-dishes. The agar medium and the herbicide concentration were prepared similar to those mentioned in the seed germination test. Seedling of R and S populations was grown in trays, at the 2-3 leaf stage, five seedlings of R and S were transplanted into Petri-dishes and placed in the greenhouse adjusted at 60% relative humidity, 12/6^{o}C, 10/14 h day/night. The efficacy of herbicide consisted of visual rating for survival seedling growth of new root and shoot 15 days after transplanting. Based on visual assessments, resistance population was 2.39 times more resistance than the S population to clodinafop-propargyl. R and S populations were susceptible to mesosulfuron-methyl+iodosulfuron (no different between ED_{50} values).

The quick test (filter-paper based test) was done in 4.5 cm in diameter Petri-dish. Five seeds of R and S populations were sown in Petri-dishes after two layers of filter paper placed in. Water was subsequently added when needed. To maintain the humidity, an amount of 30g of fine sand was added into Petri-dish after the seed germination started. Herbicides rates were 0, 12, 24, 48, 96, 192 and 384g a.i

ha^{-1} for clodinafop-propargyl; 0, 10.12, 20.25, 40.5, 81, 162 and 324g a.i ha^{-1} for pinoxaden; 0, 2.25, 4.5, 9, 18, 36 and 72g a.i ha^{-1} for mesosulfuron-methyl; 0, 4.68, 9.37, 18.75, 37.5, 75 and 150g a.i ha^{-1} for pyroxsulam. Herbicides were applied outdoors at the recommended growth stage (BBCH 13-14) arranged in an area of 10 m^2. Herbicide application was done by a rechargeable backpack sprayer (MATABI) equipped with Tee-Jet nozzles with application volume of 250 L ha^{-1} at 303.975 kPa and boom height of 50 cm from the weed. The experiment was arranged in CRD with six replicates of each herbicide rate and the whole experiment was repeated four times. The levels of injury and plant height were recorded a day before treatment and at 1, 3, 5, 7, 14, 21, and 28 days after treatment (DAT). ACCase-inhibiting herbicides symptoms appeared on both S and R populations after 14 and 21 days after treatment, respectively. In general, a lower dose of both herbicides caused interveinal bleaching (whitening) and at high dose, the plant was turned yellow. For ALS-inhibiting herbicide, 50% symptom in S and 30% in R were observed on the 14 and 28 DAT, respectively. The average RI calculated in this test was 2.89, 2.75, 2.75 and 3.27-fold for clodinafop-propargyl, pinoxaden, mesosulfuron-methyl and pyroxsulam, respectively.

The greenhouse test was conducted according to the herbicide resistance action committee (HRAC) rules. Five seeds from R and S populations were sown in pots (10 x 15 cm) containing a potting mix (field soil, sand, and peat) in a 1:1:1 ratio. Pots were placed in the greenhouse (20/8^{o}C, 14/10 h day/night and 25% relative humidity) and subsequently irrigated. Later on, seedlings were thinned into two plants per pot. Herbicides were applied and the spray condition was similar to that mentioned above in the Petri-dish assay. To assess the herbicide efficacy, R and S plants were harvested above ground at the 28th day of herbicide application, plants were dried in an oven at 65^{o}C for 72 h. The average of RI's in this test were 5.59, 10.86, 12.08 and 12.72-fold for clodinafop-propargyl, pinoxaden, mesosulfuron-methyl and pyroxsulam, respectively.

For the molecular test, plant materials for ALS and ACCase analysis from R and S populations were harvested above ground from plants survived the recommended herbicide dose in the four weeks after treatment. DNA extracted from R and S populations through CTAB protocol. PCR was conducted using a Bio-Rad thermo-cycler; the PCR product was visualized on 5% agarose gel with 2,000bp markers and sequenced. The nucleotide sequence of R and S populations has been deposited in the gen-bank database, accession numbers were received. A single amino acid replacement at Glutamine (Gln), Phenylalanine (Phe), Valine (Val), Serine (Ser), Threonine (Thr) and Glycine (Gly) was identified to confer cross-resistance to ACCase-inhibiting herbicide.

In conclusion, the greenhouse test is still the most commonly used test for determining resistance to herbicides because it is parallel to plant response in the field and is liberated of the mode of action. The results of the agar-based test are rarely compared with those of the greenhouse test because the herbicide doses in Petri-plate test and agar-based tests are much lower than recommended field doses. Many quick tests are specific to the herbicide mode of action or weed species, molecular-based tests are specific for the target-site resistance mechanism. Therefore, selecting the appropriate test is critical. Consistency, efficiency, and cost of the test are major considerations for commercialization. The partnership and communication between university, research institutes, herbicide companies, and farmers are of great importance for the detection of herbicide resistance weed and planning for the management program.

GENİŞLETİLMİŞ ÖZET

Tarım alanlarında, ürün verimi ile yabancı ot mücadelesi arasında doğru orantılı bir ilişki bulunmaktadır. Çünkü yabancı otların zararı ancak verimde görülmektedir. Son yıllarda, herbisitler kolay uygulanabilir ve hızlı ulaşılabilir olmaları, ve de kısa sürede etki göstermeleri nedeniyle yabancı otların mücadelesinde üreticiler tarafından en fazla tercih edilen tarımsal kimyasallardır. Ancak, aynı etki mekanizmalı herbisitlerin sürekli ve yoğun bir şekilde kullanımı, yabancı otlarda direnç oluşumuna neden olmaktadır. 2019 yılının başında, dünyada yaklaşık 500 biyotıpta direnç oluşmuştur. Bugüne kadar, 45 civarında yabancı ot biyotipinin ACCase (Asetil CoA Karboksilaz) inhibitörü herbisitlere ve 160 yabancı ot biyotipinin ALS (Asetolaktat Sentetaz) inhibitörü herbisitlere karşı direnç oluşturduğu tespit edilmiştir. Yabancı otlarda herbisitlere karşı oluşan bu direncin belirlenmesinde uluslarası düzeyde kullanılan farklı direnç belirleme yöntemleri kullanılmaktadır. Bu yöntemlerin, testlenecek yabancı ot türüne ve herbisitin ait olduğu kimyasal grubuna göre doğru olarak seçimi ve bu yöntemlerin etkinlik ve ekonomi açısından birbirleriyle kıyaslanması kullanılabilirliği uygulamaların açısından son derece önemlidir. Bu nedenle bu çalışmada, buğday üretim alanlarında ana zararlı olan Kısır Yabani Yulaf (*Avena sterilis* L.) popülasyonlarının ACCase (clodinafop-propargyl+cloquintocet-mexyl and pinoxaden+cloquintocet-mexyl) ve ALS inhibitörü herbisitlerine (Mesosulfuron-methyl+iodosulfuron-methyl-sodium ve pyroxsulam+cloquintocet-mexyl) karşı oluşturduğu direncin belirlenmesinde kullanılan farklı yöntemlerin kullanılması ve karşılaştırılması amaçlanmıştır.

Çalışmada, Kozan İlçesi-Adana İlinde bir buğday tarlasında bulunan ve daha önceden dirençli olduğu belirlenen Kısır Yabani Yulaf (*Avena sterilis* L.) popülasyonlarına ait tohumlar toplanarak yapılan tüm denemelerde kullanılmıştır. Herbisit uygulanmadığı bilinen alanlardan ise hassas Kısır Yabani Yulaf denemelerde kullanılmak üzeri tohumları toplanmıştır. Toplama işlemi 2016 yılı Mayıs-Haziran aylarında yapılmıştır. Dirençli ve hassas tohumlar kavuzlarından ayrılarak, kese kağıtları içerisinde +4°C'de dormansilerinin kırılması amacıyla muhafaza edilmiştir (yaklaşık 60 gün). Toplanan örneklerde çalışmada seçilen herbisitlere karşı direnç olup olmadığı ise hızlı testler (Filtre kağıdı ve Agar), sera

testleri ve moleküler testler ile belirlenmiştir. Tüm çalışma boyunca uygulanan testler aşağıda sırasıyla verilmiştir.

Hızlı testlerden ilki olan agar testi, petri kaplarında, bitki agarı (0.9g/L) ortamında, altı tekerürlü olmak üzere iki kez kurulmuştur. Mikrodalga fırında eritilen agar, herbisit eklemeden önce yaklaşık 35-40°C' ye kadar soğutulmuştur. Soğuyan agar içerisine clodinafop-propargyl+cloquintocet mexyl 0, 1.2, 2.4, 4.8, 9.6, 19.2 ve 38.4 µM; pinoxaden+cloquintocet -mexyl 0, 0.225, 0.45, 0.9, 1.8, 3.6 ve 7.2 µM; mesosulfuron-methyl+iodosulfuron-mexyl 0, 0.15, 0.3, 0.6, 1.2, 2.4 ve 4.8 µM ve pyroxsulam+cloquintocet-mexyl 0, 0.37, 0.75, 1.5, 3, 6 ve 12 µM eklenmiştir ve dokuz cm çapındaki cam petri kaplarına 20 ml agar dökülmüştür. Agar dökülen petri kaplarına, dirençli ve hassas populasyonlardan on adet tohum ekilmiştir. Petri kapları, 15 gün boyunca 10°C' de buzdolabında bekletilmiştir. Yapılan denemeler sonucunda, kontrolde dirençli ve hassas tohumların %95'in üzerinde çimlendiği saptanmıştır. Clodinafop-propargil, mezosülfüron-metil ve pirokssulam uygulamalarında, her iki populasyon %20'den fazla çimlenme göstermiştir. Pinoxaden uygulamalarında ise dirençli ve hassas populasyonların %10'dan daha az oranda çimlendiği belirlenmiştir. Çimlenen tohumların ise hipokotil ve radikula uzunlukları baz alınarak, ED_{50} değerleri hesaplanmıştır. Clodinafop-propargyl, pinoxaden, mesosulfuron-methyl ve pyroxsulam uygulamalarında hipokotil direnç indeksleri (RI) sırasıyla 3, 3, 2.10 ve 1.58 kat; radikula direnç indeksleri ise 1.99, 5.24, 2.67 ve 3.32 kat olarak tespit edilmiştir.

Seradaki biyotest denemelerinde dirençli ve hassas Kısır Yabani Yulaf popülasyonların tohumları viyollere ekilerek çimlenen bitkiler 2-3 yaprak dönemine geldiği zaman, beş adet dirençli ve hassas bitki viyollerden sökülerek kökleri temizlenmiş ve agar ortamına dikilerek sera ortamında (yaklaşık %60 nem, 12/6^{o}C, 10/20'ye (gündüz/gece)) 15 gün boyunca gelişimi saptanmıştır. Denemenin sonunda (15. gün) hayatta kalan bitkilerin kök ve gövde gelişimleri dikkate alınarak herbisitin etkisi görsel olarak değerlendirilmiştir. Görsel değerlendirmeler sonucunda, dirençli populasyonun, clodinafop-propargil uygulamalarında direnç endeksi 2.39 kat olup, mesosulfuron-methyl+iodosulfuron uygulamalarında bu herbisite karşı direnç oluştumadığı tespit edilmiştir ED_{50} değerleri arasında fark bulunmamıştır.

Çalışmada uyguladığımız üçüncü hızlı test, petri kapları ve filtre kağıdı kullanılarak yapılmıştır. Hızlı test denemelerinde, 4,5 cm çapındaki petri kaplarına iki kat filtre kağıdı yerleştirilmiş ve petri kaplarına dirençli ve hassas popülasyonların tohumundan beş adet konulmuştur. Denemelerde, tohum çimlendikten sonra nemin korunması amacıyla, petri kaplarına 30g elenmiş ince kum eklenmiştir. Bitkiler, 3-4 yapraklı döneme geldiği zaman 10 $m^{2'}$ lik bir alanda clodinafop-propargyl 0, 12, 24, 48, 96, 192 ve 384g a.i ha^{-1}; pinoxaden 0, 10.12, 20.25, 40.5, 81, 162 ve 324g a.i ha^{-1}; mesosulfuron-methyl 0, 2.25, 4.5, 9, 18, 36 ve 72g a.i ha^{-1}; pyroxsulam 0, 4.68, 9.37, 18.75, 37.5, 75 ve 150g a.i ha^{-1} petri kaplarına uygulanmıştır. Herbisitler, 303.975 kPa'da 250 L ha^{-1} uygulama hacmine sahip, Tee-Jet meme tipli sırt pülverazitörü (MATABI) ile uygulanmıştır. Herbisit uygulamasından önce ve sonra 1., 3., 5., 7., 14., 21. ve 28. günde herbisitin oluşturduğu simptomlar ve bitki boyları kayıt edilmiştir. ACCase inhibitörü herbisitlerin simptomları, hassas populasyonlar üzerinde 14. günde gözlemlenirken; dirençli populasyonlar üzerinde 21. Günde, ALS inhibitörü herbisitler, hassas populasyonlarda 14. günde ve dirençli populasyonlarda 28. günde %50 simptom oluşturmuştur. Çalışma sonunda, clodinafop-propargyl, pinoxaden, mezosulfuron-metil ve pyroxsulam uygulamalarında elde edilen ortalama direnç indeksi sırasıyla 2.89, 2.75, 2.75 ve 3.27 kat olarak bulunmuştur.

Sera testi ile herbisit direnci, HRAC tarafından belirlenen yönteme göre tespit edilmiştir. Sera testinde, dirençli ve hassas populasyonlardan beş adet tohum, 1: 1: 1 oranındaki (tarla toprağı, kum ve torf) saksılara (10 x 15 cm) ekilmiş ve seraya (20/8^{o}C, 14/10 saat gündüz/gece ve yaklaşık %25 nem oranı) yerleştirilmiştir. Serada çimlenen bitkiler, 3-4 yaprak döneme geldiğinde herbisit uygulamaları, herbisit dozları ve uygulamaları şekli ‘Petri Kabı Testi’ndeki gibi yapılmıştır. Herbisit uygulamasının 28. gününde toprağın üzerindeki bitkiler kesilerek hasat edilmiştir. Hasat edilen bitkiler, 65°C'de 72 saat süreyle etüvde kurutulmuştur. Clodinafop-propargyl, pinoxaden, mesosulfuron-methyl ve pyroxsulam uygulamalarında direnç indeks ortalaması, sırasıyla 5.59, 10.86, 12.08 ve 12.72 kat bulunmuştur.

ALS ve ACCase herbisitlerine direnç son alarak moleküler test yöntemiyle belirlenmiştir. Bu amaçla, dirençli ve hassas populasyonlardan toplanan dirençli ve hassas örneklerin DNA ekstraktları, CTAB protokolüne göre elde edilmiştir. Elde

edilen DNA'lar klasik PCR yöntemiyle çoğaltılmıştır. Elde edilen PCR ürünleri, %5'lik agaroz jelde koşturulmuştur. Agaroz jel sonucunda elde edilen nükleotit dizilimleri sekansa gönderilmiştir. Sekans sonuçları incelendiğinde, ACCase inhibitörü herbisitlerinin oluşturduğu mutasyon noktalarının Glutamin (Gln), Fenilalanin (Phe), Valin (Val), Serin (Ser), Threonine (Thr) ve Glisine (Gly) olduğu belirlenmiştir.

Sonuç olarak, sera testi günümüzde herbisitlere karşı direnci belirlemek amacıyla en çok kullanılan testlerden biridir. Filtre kağıdı ve agar ortamındaki testlerde herbisit dozları, önerilen alan dozlarından çok daha düşüktür. Dolayısıyla agar ortamında yapılan test sonuçları ile sera testinin sonuçları nadiren karşılaştırılır. Pek çok hızlı test, herbisit etki şekline veya yabancı ot türlerine özgüdür, moleküler testler ise herbisitlerin etki mekanizmasındaki hedef bölgeye spesifiktir. Bu nedenle uygun testi seçmek çok önemlidir. Testin tutarlılığı, etkinliği ve maliyeti kullanımının yaygınlaştırılmasında oldukça önemlidir. Herbisitlere dirençli yabancı otların tespiti ve direnç yönetim programının planlanmasında üniversitelerin, araştırma enstitülerin, herbisit üreten firmaların ve çiftçilerin ortak çalışıp aralarındaki iletişimin devamlılığı oldukça büyük önem taşımaktadır. Aşağdaki tabloda uygulanan direnç testlerinin avantajları ve dezavantajları verilmiştir.

Uygulanan Direnç Testlerinin Avantajları ve Dezavantajları

Yöntemler	Hızlı Sonuç	Den. süresi	Tüm Y.O türleri	Tarla Koşulları	Tüm Herbistler	Etki-Mek göre	Maliyet
Agar Ortamında (Tohum)	Evet	10-14 gün	Hayır	Hayır	Hayır	Hayır	Orta
Agar Ortamında (Fide)	Evet	15 gün	Hayır	Hayır	Hayır	Hayır	Orta
Filtre kağıdı	Hayır	1-2 ay	Hayır	Hayır	Evet	Hayır	Düşük
Sera Testi	Hayır	2-3 ay	Evet	Evet	Evet	Evet	Orta
Moleküler Yöntem	Çok Hızlı	2-5 gün	Evet	Hayır	Evet	Evet	Çok Yüksek

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LIST OF SYMBOLS AND ABBREVIATIONS

a.i	: Active Ingredient
ACCase	: Acetyl-CoA Carboxylase
ALS	: Acetolactate Synthase
AHAS	: Acetohydroxy Acid Synthase
ANOVA	: Analysis of Variance
bp	: Base Pair (s)
cm	: Centimeter (s)
DAT	: Days After Treatment
°C	: Degree of Celsius
DNA	: Deoxyribonucleic Acid
EC	: Emulsifiable Concentrate
ED_{50}	: Dose Required to Kill 50% of Test Populations
EWRS	: European Weed Research Society
g	: Gram (s)
GPS	: Global Positioning System
ha	: Hectare (s)
HRAC	: Herbicide Resistance Action Committee
IPM	: Integrated Pest Management
IWM	: Integrated Weed Management
MoA	: Mode of Action
Pa	: Pascal
PCR	: Polymerase Chain Reaction
PRE	: Pre Emergence Herbicide
POST	: Post Emergence Herbicides
%	: Percentage
SE	: Standard Error

VLCFA : Very Long Chain Fatty Acid

WSSA : Weed Science Society of America

Wt/v : Weight by Volume

1. INTRODUCTION

Weeds are among the major pests that cause significant yield losses. They compete with crop plants for water, sunlight, nutrients, space and host different diseases and insects (Oerke and Dehne, 2004; Li *et al.*, 2017b). World-widely, weeds can cause 35% crop yield losses on an average (Oerke, 2006).

Sterile wild oat (*Avena sterilis* L.) is an annual grass weed species, hexaploid, autogamous, competitive weed species infesting in range of cereal crops in the Mediterranean region, and causes significant yield losses (Dhima and Eleftherohorinos, 2001; Barroso *et al.*, 2006; Fernandez-Moreno *et al.*, 2016; Castillejo-Gonzalez *et al.*, 2019). *A. sterilis* is very common and the most prevalent winter annual grass weed in winter wheat fields of Çukurova region situated in the basin of the Mediterranean Sea, which considered to be one of the main agricultural areas of Turkey (Kadıoğlu, 1989). Uygur *et al.* (1993) reported the occurrence of wild oats in 86% of the surveyed fields in the Çukurova region in the mid-1980s and in almost all fields in the early 1990s'. Lack of crop rotation combined with several changes in agronomic practices has contributed to the spread of sterile wild oat worldwide (Beckie *et al.*, 2004; Yücel, 2004). Yield reduction in wheat, barley, canola, oat, maize and alfalfa due to *A. sterilis* interference was estimated to be more than 70%, depending on many factors such as crop-seeding rate, season and infestation level (Dhima *et al.*, 2010; Armengot *et al.*, 2017; Bajwa *et al.*, 2017). The economic thresholds of wild oat in Turkey estimated as 3 to 5 plants/m² (Kadıoğlu, 1989). Uncontrolled sterile wild oat can significantly reduce the quality of cereal crops and harvesting efficiency (King, 2007).

Since the introduction of synthetic herbicides in late 1940 (Shen *et al.*, 2009; Busi *et al.*, 2018), herbicides have been an inevitable tools for weed control in modern crop production systems because they provide an economical and effective way to manage weeds (Shaner, 2014; Duke *et al.*, 2019). The use of

herbicide for weed control replaced several cultural, physical, and mechanical weed control practices and significantly contributed to the high productivity of global agriculture (Retzinger and Mallory-Smith, 1997; Burns *et al.*, 2018). Nowadays, herbicides are important components in integrated weed management (IWM) practices and in intensive agriculture (Petit *et al.*, 2010b; DeDecker *et al.*, 2014; Harker *et al.*, 2016; Uygur, 2017). However, the repeated application of herbicides with the same mode of action combined with global changes in modern agricultural systems (intensive cropping systems, short rotations, etc.) has resulted in the evolution of herbicide resistance in agricultural weeds (Preston and Powles, 2002; Reddy and Nandula, 2012; Panozzo *et al.*, 2017).

Blackman (1950) and Harper (1956) predicted the evolution of herbicide resistance in weeds shortly after the appearance of herbicides into the global market. The first case of herbicide resistant-weed recorded in Canada dated back in 1957 in wild carrot (*Daucus carota* L.) against auxinic herbicide 2,4-D (Timmons, 2005; Beffa *et al.*, 2019). Afterward, during late 1970s new case of herbicide resistant-weed was reported in the USA in a biotype of common groundsel (*Senecio vulgaris* L.) against PSII-inhibitors (herbicide inhibiting the electron transport in photosystem II) (Ryan, 1970; Duke and Heap, 2017). In May 2019, the international survey of herbicide-resistant weeds recorded 500 unique cases (species x site/mode of action, MoA) of herbicide-resistant weeds globally, representing 256 weed species (149 dicotyledonous and 107 monocotyledonous) for 23 of the 26 known herbicide sites of action to 167 different herbicides in 93 crops in 70 countries (Heap, 2019).

Acetyl-CoA carboxylase (ACCase) and acetolactate synthase (ALS)-inhibiting herbicides are extensively used to control grass weeds including *Avena* spp., which become highly resistant to these herbicides and reported in many locations (Vercellino *et al.*, 2018). The most widespread resistance cases of *A. sterilis* were reported in ACCase inhibitors (Cocker *et al.*, 2000; Cruz-Hipolito *et al.*, 2011). First resistance case to ACCase herbicide (diclofop) was detected in

ryegrass (*Lolium rigidum* Gaud.) in 1982 in Australia (Heap and Knight, 1982), later in 1989, resistance was reported for the first time in *A. sterilis* (Kaloumenos *et al.*, 2012). In addition, weeds resistant to ALS-inhibiting herbicides was observed first in 1987 (Mallorysmith *et al.*, 1990), reported first time in *A. sterilis* in 1997 in France (Heap, 2019). In Turkey, sterile wild oat evolved resistance for the first time in 1997 to ACCase, clodinafop-propargyl, diclofop-methyl, fenoxaprop-P-ethyl, and tralkoxydim (Uludag *et al.*, 2007), while first resistance case to ALS, pyroxsulam was reported in 2008 (Uygur *et al.*, 2014). Since then, 14 resistance cases in *A. sterilis* have been documented in 9 different countries to 3 different modes of action MoA (ACCase, ALS and unknown group Z) of herbicides (Heap, 2019). Both target-site and non-target-site herbicide resistance mechanisms have been detected in *A. sterilis* (Powles and Yu, 2010). In general, the evolution of HR in weeds has a significant impact on the sustainability of crop production all over the world (Brunton *et al.*, 2019).

Various herbicide resistance diagnostic tests have been used for screening seeds from suspected resistance plants. Petri-dish tests involve germinating seeds on agar medium or filter paper with herbicide (Heap, 1994). Other resistance tests include germinating seeds in pots treated with herbicides, which needs more resources, space and time (Tal *et al.*, 2000; HRAC, 2019). Other tests such as germinating pollen in agar (Richter and Powles, 1993) or growing tillers in herbicide medium (Letouzeâ *et al.*, 1997) have been described. These tests have been used on grass weeds such as ryegrass (Kuk *et al.*, 2000), Japanese foxtail (*Alopecurus japonicus*) (Yang *et al.*, 2007), monochoria (*Monochoria vaginalis*) (Kuk *et al.*, 2003) and green foxtail (*Setaria viridis*) (Beckie *et al.*, 1990). Despite the worldwide distribution of herbicide-resistant weeds, there is relatively limited research conducted on comparing the methods used for detecting herbicide-resistant weed populations.

The research was aimed mainly to:

1. Determine the resistance level of sterile wild oat (*Avena sterilis* L.) populations against clodinafop-propargyl, pinoxaden, mesosulfuron-methyl+iodosulfuron-methyl-sodium and pyroxsulam+cloquintocet-mexyl.
2. Describe and compare the reliability and validity of various tests used for detecting herbicide resistance under greenhouse and laboratory conditions.
3. Identify the best and cost-effective test for herbicide resistance detection.

2. LITERATURE REVIEW

2.1. Definition and Mechanism of Herbicide Resistance

Herbicide resistance (HR) is inherited ability of weed to survive and reproduce following exposure to a dose of herbicide that would normally result in effective weed control (WSSA, 1998; Moss, 2017). In the agricultural situations, Heap and Lebaron (2001) defined HR as the evolved capacity of previously herbicide-susceptible weed to survive a herbicide and complete their life cycle when the herbicide is applied at the recommended field rate. It is important to note that weeds can evolve cross-resistance and multiple-resistance. The term 'cross-resistance' used to describe when a single resistance mechanism confers resistance to two or more herbicides (same chemical class, or different) within individual plant, where the term 'multiple resistance' is used where more than one resistance mechanisms occur within an individual plant (Hall *et al.*, 1994; Rubin, 1997; De Prado and Franco, 2004).

Herbicides classification according to their site of action is comparatively better as herbicide resistance can be handled and managed more properly and effectively (Forouzesh *et al.*, 2015). There are three classification systems for the herbicide site of action. First, the WSSA classification system was established in 1997 based on Arabic numbers (Mallory-Smith and Retzinger, 2003), which is only used in the USA and Canada. Second, the Australian classification system was created in the early 1990s based on letters and it is only used in Australia. Third, the classification system of herbicide resistance action committee (HRAC) that formed in 1989 and used in all other countries (Retzinger and Mallory-Smith, 1997), HRAC classification is based on the letter (Figure 2.1) but not similar to those of the Australian classification system.

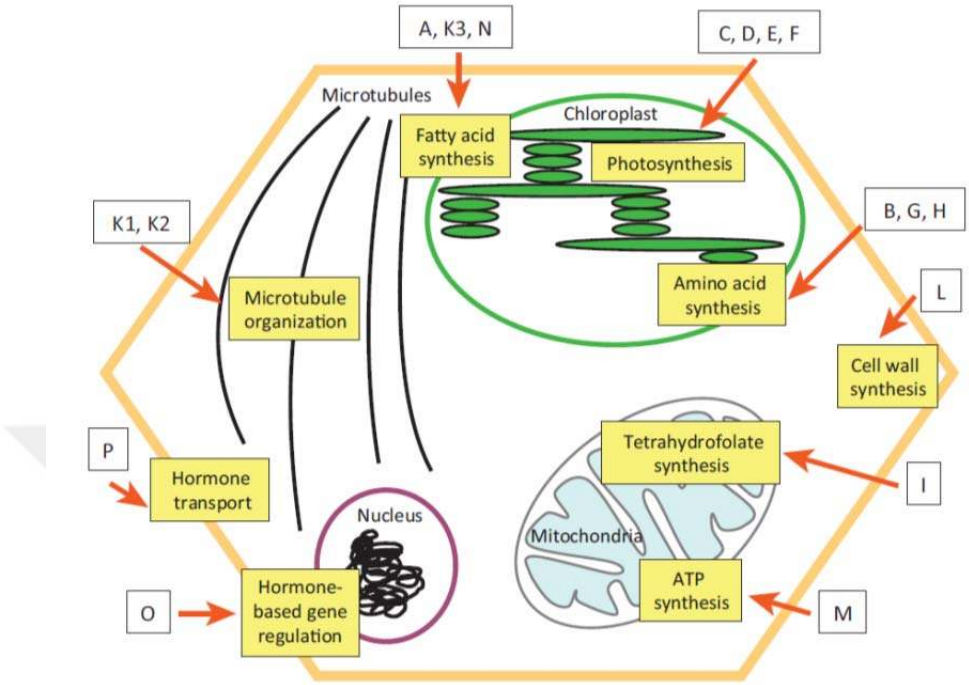

Figure 2.1. Herbicide site of actions and their classification according to the herbicide resistance action committee (HRAC) classification system (Delye *et al.*, 2013).

Mechanisms of HR in weeds can be divided into two main categories (Delye *et al.*, 2015; Feng *et al.*, 2016). First, target-site resistance (TSR) mechanism resulting from the alteration or increased expression of the herbicide-binding site (Figure 2.2) that prevents or reduces the ability of a herbicide to bind to the target site. Second, non-target-site resistance (NTSR) is due to diverse mechanisms, e.g., decrease in herbicide uptake or translocation in the plant, or enhanced metabolism (Yuan *et al.*, 2007), and reduce the amount of herbicide reaching a target site. However, NTSR recognized as being of main significance in resistance to the ACCase, ESPS, ALS and PSII-inhibitors (Delye, 2013; Delye *et al.*, 2013).

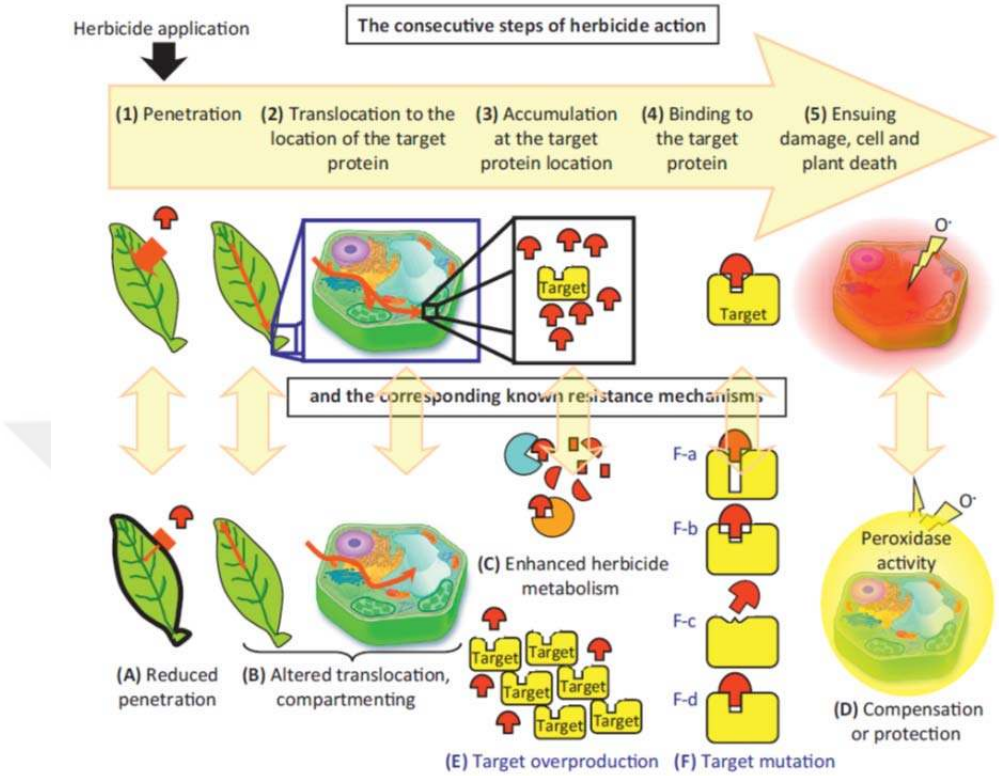

Figure 2.2. Mechanisms of herbicide resistance, target-site resistance (TSR) and non-target site resistance (NTSR;) (Delye *et al.*, 2013).

2.2. Herbicides Resistance in *Avena sterilis* L. in Turkey and in the World

Avena sterilis populations evolved herbicide resistance (R) worldwide to one or more herbicides from a different mode of action in various cropping systems (Heap, 2019). To give information to the readers, recent studies on resistance cases of *A. sterilis* against ALS and ACCase-inhibiting herbicide are stated below.

Maneechote et al. (1997), found resistance level 6-fold in *Avena sterilis* populations against diclofop-methyl. Absorption and translocation of [^{14} C] diclofop-methyl applied at two-leaf stage plants were similar in R and susceptible (S) populations; the rate of metabolism was increased 1.5-fold in the R compared to the S populations.

In the USA, Mengistu *et al.* (2003), studied the diversity of resistance among *Avena* spp. collected in 1964 and 2000 and screened with imazamethabenz, difenzoquat, diclofop, fenoxaprop-P, sethoxydim, and tralkoxydim. 43% of the samples collected in 1964 were S to these herbicides. Whereas, only 9% collected in 2000 were S. The evolution of resistance in 2000 compared with 1964 increased for all the herbicides. The number of R plants increased faster for the diclofop and fenoxaprop-P than for the sethoxydim and tralkoxydim, which suggested that the populations had evolved cross-resistance.

In Turkey, Uludag *et al.* (2007), reported resistance cases in seven *A. sterilis* populations, AKR1; AKR2; DZC; GKY1; GKY3; KMP; KMT, to fenoxaprop. The resistance indices were between 2.41-and 48.0-fold. All populations were also evolved cross-resistance to clodinafop. AKR2 had cross-resistance to all ACCase herbicides, the KMT was resistant to all APPs and GKY1 showed resistance only to fenoxaprop.

In Australia Liu *et al.* (2007), studied fenoxaprop resistance in *A. sterilis* ssp. *ludoviciana* Durieu on the molecular basis. He found five mutations in the ACCase gene, Ile-1,781-Leu, Trp-1,999-Cys, Trp-2,027-Cys, Ile-2,041-Asn, and Asp-2,078-Gly. An allele-specific PCR test was used to determine the prevalence of these five mutations in wild oat population's suspected of ACCase resistance. These mutations are very likely to confer resistance to any grass weed species under selection imposed by the extensive agricultural uses of the herbicides.

In Iran, Kashani *et al.* (2007), conducted the greenhouse and laboratory tests to determine resistance in *Avena luduviciana* populations to fenoxaprop-p-ethyl. The tested population showed resistance to fenoxaprop-p-ethyl in both experiments with different levels. The seed bioassay provides a simple, quick, low-cost and precise method for identifying sterile wild oat resistant to ACCase herbicides.

Uludag *et al.* (2008), studied five resistant populations of wild oat collected from wheat and lentil fields in the Pacific Northwest. He found 2 to 24-

fold resistance to fenoxaprop, diclofop, and quizalofop compared with the susceptible biotype. Some biotypes were resistant to the sethoxydim and clethodim. R2 biotype was 35-and 16-fold resistant to tralkoxydim and pinoxaden, respectively. The levels of resistance and cross-resistance patterns varied among biotypes.

Travlos *et al.* (2011), conducted studies on the occurrence and distribution of herbicide-resistant *A. sterilis* in Greece in 2009. In total, 104 samples were screened in a field experiment with several ACCase herbicides. Most of the samples (89%) were resistant or developing resistance to diclofop. In the pot experiments, some of the samples showed a very high level of diclofop resistance >28.6, while cross-resistance was common. Other ACCase herbicides, tralkoxydim, and pinoxaden remained effective for 86 and 92% of the tested samples, respectively. All biotypes treated with mesosulfuron + iodosulfuron were susceptible (97%).

In Turkey, Tursun (2012), determined the resistance of *A. sterilis* biotype to fenoxaprop-p-ethyl in a laboratory using seeds on 1.3% agar with concentrations of 0, 7.68, 15.36, 30.72, 61.44, 122.88 and 243.76 µM. The biotype had 14-fold and 10-fold resistance levels in radicle and hypocotyl, respectively.

In Greece, Papapanagiotou *et al.* (2012), observed unsatisfactory control of *A. sterilis* population's when treated with fenoxaprop-p-ethyl and clodinafop-propargyl. Less than 80% of the treated plants were killed by the application of the four times the recommended dose of fenoxaprop-p-ethyl and clodinafop-propargyl. In the dose-response study, the population was cross-resistant to clodinafop-propargyl, tralkoxydim, and pinoxaden. All the populations were effectively controlled by the recommended field dose of imazamox and mesosulfuron-methyl + iodosulfuron methyl-sodium.

In Western Australia, Ahmad-Hamdani *et al.* (2012), studied on four *Avena* spp. populations to determine the resistance levels of aryloxyphenoxypropionates, cyclohexanediones and phenylpyrazoline herbicide

(pinoxaden). The resistance index (RI's) from the dose-response study (greenhouse experiment) in four R populations showed high-level of resistance to diclofop but varied to other phenylpyrazoline herbicides tested. It is obvious that *Avena* spp. populations from another part of Western Australia had resistance to a number of ACCase-inhibiting herbicides.

In Canada, Beckie *et al.* (2012b), investigated on target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms conferring ACCase and ALS resistance in 16 *Avena* spp. populations. Seven ACCase mutation points were detected in Ile1781Leu, Trp2027Cys, Asp2078Gly, Trp1999Cys, Ile2041Asn, Cys2088Arg, and Gly2096Ser. Two populations confirmed with ALS target-site mutation, Ser653Thr, and Ser653Asn. This was the first report across the world of ALS target-site mutations in *Avena* spp.

Adamczewski *et al.* (2013), studied 34 samples of wild oat biotypes in the greenhouse and determined the resistance to herbicides. Three R biotypes (R3, R4, and R5) developed resistance to iodosulfuron and mesosulfuron. Biotype R1 developed multiple resistance to pinoxaden and iodosulfuron + mesosulfuron. Two biotypes of wild oat (R1 and R4) revealed of mechanism to ACCase associated with target-site mutation.

Ahmad-Hamdani *et al.* (2013), investigated the biochemical basis of resistance in *Avena* spp. to diclofop-methyl. Uptake and translocation of [^{14}C]-diclofop were the same in the R and S biotypes. Non-target-site based mechanism of enhanced rate of diclofop acid metabolism confers resistance in R biotype. On the other hand, the high-performance liquid chromatography (HPLC) showed the major diclofop metabolites in R is similar to that of wheat, suggesting resistance in individuals of population R involves a wheat-like detoxification system mediated by cytochrome P450 monooxygenases.

In Turkey, Uygur *et al.* (2014), determined clodinafop-propargyl resistance in *Avena sterilis* in 2011-2012 by quick-test and greenhouse experiment. In 80 populations collected in 2011 and tested, 49% was found resistance (RI >2), 41%

was indicated low resistance (RI <2) and 10% was not resistance (RI <1). In 2012, 62 populations collected and tested, 74% has resistant and 26% has been considered risky. Herbicide resistance map of *A. sterilis* was created.

Travlos (2013), mentioned that the fecundity and competitive ability of diclofop and fenoxaprop resistant *A. sterilis* biotype compared with S biotype was similar. There were no differences in seed germination and seedling vigor among two biotypes at any temperature regime. The growth of the R and S biotype was similar on the basis of plant height, canopy area, seed production, and plant biomass.

In Montana, USA, Keith *et al.* (2015), found resistance cases in *Avena* spp. biotypes against four herbicides from three different modes of action: tralkoxydim (ACCase), imazamethabenz and flucarbazone (ALS), difenzoquat (growth inhibitor). In quantifying resistance levels of the same biotype in greenhouse studies to triallate, pinoxaden and paraquat. He found the resistance level of 17.5- and 18.1-fold to triallate, 3.6- and 3.7-fold to pinoxaden, and 3.2-fold to paraquat.

In Greece, Papapanagiotou *et al.* (2015), reported resistance cases in *A. sterilis* populations against fenoxaprop and clodinafop. In molecular studies, he confirmed six mutation points: Ile-1781-Leu, Trp-1999-Cys, Trp-2027-Cys, Ile-2041-Asn, Asp-2078-Gly, and Cys-2088-Arg in ACCase gene sequence from 24 resistant populations and two populations reveal any known ACCase mutation points. This may due to the presence of non-target-site resistance mechanisms.

Busi *et al.* (2016), studied on the phenotypic variation, in response to plant survival at low-dose diclofop-methyl. A significant difference was detected between the selected progenies and parent plants, with a 2-fold diclofop-methyl resistance and cross-resistance to ALS-inhibiting herbicides. The response of self-pollinated *A. fatua* to low-dose herbicide selection is marginal, and it is much lower than other cross-pollinated grass species. Cross-pollination rate and genetic variation are identified as possible drivers affecting the contrasting capacity of

Avena versus other grass weed to respond to herbicide selection and the subsequent likelihood of resistance evolution at low herbicide dose usage.

In Western Australia, Owen and Powles (2016), conducted a random survey in 2010 across 14 million hectares to monitor the change in HR levels and compared the results with previous studies done in 2005. 48% of *Avena* spp. populations showed resistance to diclofop, fenoxaprop, pinoxaden, tralkoxydim, and sethoxydim, which was lower than that found in 2005 (71%). In the study, resistance to mesosulfuron, and imazamox + imazapyr was reported for the first time in Western Australia.

In Iran Sasanfar *et al.* (2017), examined 12 putative R and one S populations of *Avena ludoviciana* to determine the level of resistance and patterns of cross-resistance to clodinafop, sethoxydim, and pinoxaden. Plant biomass and coleoptile lengths were measured in whole-plant and seed bioassays, respectively. Result of whole-plant assay revealed that all 12 populations showed resistance to clodinafop with resistance ratios (R/S) between 1.76 to >47.04. Clodinafop-resistant populations showed cross-resistance to sethoxydim and pinoxaden with R/S values 10.73 to 40.29. The result of the seed germination test was similar to that obtained from the whole-plant, suggesting the seed test was a low-cost, rapid for detecting-resistant biotypes.

2.3. Methods for Detecting Herbicide Resistance

Herbicide resistance tests start by field surveys, sampling, screening, data collection and interpretation of results and delivering the information to the growers (Beckie *et al.*, 2000). Several methods for testing herbicide resistance were described by Corbett and Tardif (2006), and Burgos (2015) to help researchers and farmers to manage herbicide-resistant weeds.

2.3.1. Quick-Tests for Detecting Herbicide Resistance

Due to the increasing the number of herbicide-resistant weeds, the request for rapid diagnostic tests has been increased. A Petri-dish test was used to evaluate the herbicide-resistance of large samples in a short time period and less space. Good seed quality is needed for the Petri-dish test. The test starts with incubating seeds in the Petri-dish after filter paper placed in and lined with various herbicide concentrations. The coleoptile elongations are used as indicators of resistance within two weeks of incubation (Corbett and Tardif, 2006). Other quick methods are slightly varied from the above-mentioned test. The seeds were germinated in Petri-dishes containing herbicide-amended agar media and placed in a growth chamber or in the greenhouse. Coleoptile lengths were measured 7-14 days. The test was able to identify different resistance patterns (Burgos, 2015). Recently, a resistance in season quick (RISQ) test was developed to determine the resistance of various herbicides in grass weed species. The seedling was grown in trays and transplant into the Petri-dish containing agar amended with herbicide and evaluate the efficacy of herbicide by a visual rating of surviving plants. The test needs bench-top and supplementary light (Burgos *et al.*, 2013).

Burnet *et al.* (1994), described a test to determine ALS herbicide resistance in ryegrass (*Lolium rigidum* L.). Agar (0.6%) was powered in 9-cm Petri-dishes (20 ml per dish) and allowed to solidify. Seeds were placed uniformly over the agar surface; dishes were sealed with polyethylene film to prevent moisture loss and placed in an incubator illuminated with fluorescent lights (photoperiod, 12 h; temperature, 20^{o}C dark/light). Five days later, the seeds were counted to determine the total number of seeds, the proportion in which the coleoptile had emerged, and the proportion in which the first leaf had emerged over 1 cm from the coleoptile. LD_{50} was determined by regression of the % mortality against the log of herbicide dose.

Murray *et al.* (1996), developed a rapid test to detect the clodinafop, clethodim and tralkoxydim resistance in wild oat. The seeds were germinated on

Plexiglas boxes contained on a 1-cm-thick agar medium with herbicide. Plates were left in the dark for 5 days at 21°C. Coleoptile lengths were measured 5 DAT. The test was replicated three times, with new plates and new agar. For each concentration, a mean coleoptile length was calculated and dose-response curves were studied.

Bourgeois *et al.* (1997), developed seed bioassay for rapid identification of resistance to fenoxaprop-P and sethoxydim. Presence or absence of resistance in wild oat was based on coleoptile and hypocotyl length of seedlings on an agar medium amended a discriminatory concentration of herbicide.

LetouzÉ and Gasquez (1999), developed a quick test to determine resistance to ACCase herbicide in *Alopecurus myosuroides* and *Lolium* spp. based on the difference in coleoptile length of R and S. *A. myosuroides* exposed to 6 mg L^{-1} fenoxaprop-P acid solution for 6 days in a plastic box. Coleoptile lengths of S were shorter than the R. Seedlings of *Lolium* spp. at 10 mg L^{-1}, diclofop acid concentration; coleoptile lengths of S and R were shorter and longer than 20 mm, respectively. For both species, the coleoptile length seems to distinguish between two HR biotypes.

Kim *et al.* (2000), developed a rapid test to evaluate R and S biotypes of *Echinochloa colona* to propanil or fenoxaprop-P at all growth stages. Pre-germinated seed test for fenoxaprop-P on 1.0% agar medium containing a range of herbicide concentrations and kept under humid conditions. For propanil, seeds were placed on moist filter paper placed in the Petri-dish. For the two tests, seedling length and fresh weight were measured 7 DAT. For young plants, 4-leaf to 1-tiller, shoots, and roots were trimmed and placed in 20-ml glass tubes containing 0.2% (wt/v) agar with a range of herbicide concentrations. Shoot elongation and fresh weight were recorded 7 DAT. For larger plants, tillers were removed from the mother plants and evaluated as described for young plants. In three tests GR_{50} values for shoot length and fresh weight were used to distinguish R and S biotypes.

These tests are provided reliable and quick, covering various growth stages from seed to flowering.

Letouzé and Gasquez (2000), developed a quick test to determine ACCase herbicides in *Alopecurus myosuroides*. The test is based upon germination of pollen in a medium amended ACCase herbicide. Pollen was germinated in 0.25% agar medium, containing 200 mg L^{-1} $CaNO_3$, 100 mg L^{-1} H_3BO_3, 200g L^{-1} sucrose. At 25°C, the medium provided a mean germination rate of 85% within two hours. If the pollen germinates very fast plants were classified highly resistant.

Boutsalis (2001) developed Syngenta Quick-Test, (QT) for detecting grass weed resistance to ACCase and ALS herbicides. He cut the growing point of seedling plants and transplant into pots in the greenhouse. The plants were applied with herbicides and evaluate the efficacy after one week of transplanting. In greenhouse experiments using seeds, known herbicide-resistant grass weeds to the ACCase and ALS herbicide was verified by the QT, and a similar result was observed from both experiments. Resistance was evaluated in less than 4 weeks. QT suggests a possibly suitable for the in-season assay of surviving grass weeds.

Cirujeda *et al.* (2001), described the seed-based test to detect the resistance of tribenuron-methyl in *Papaver rhoeas* L. The seeds were germinated on 35 ml of a 1.3% agar medium containing 2g KNO_3 L^{-1} in 8.5 cm Petri dishes in a growth chamber with additional light. At a concentration of 0.24 µM tribenuron-methyl, the S plants stopped growth after the cotyledon stage, whereas R plants continued to produce new leaf. 14 days were needed to finish the test, sometimes plants grew slowly and developed smaller leaves, 17 days required to complete the test.

Walsh *et al.* (2001), studied in the populations of wild radish (*Raphanus raphanistrum* L.) resistant to chlorsulfuron. Plants were collected and transplanted into pots and screened using the Quick-Test method. This test effectively identified the resistant of wild radish. Dose-response tests with the seed of the same populations confirmed their resistance and validated the use of the Quick-Test for a dicot species.

Delye *et al.* (2002b), described a rapid seed-bioassay to assess sethoxydim sensitivity of *Setaria* sp. Seeds were placed on filter paper placed at 0.7 cm-diameter glass tubes in 13.5-cm-diameter glass dishes containing 60 ml sethoxydim solution (33 μM). Plates were incubated for 72 h at 27°C in darkness. A seedling was considered sensitive if the length of coleoptile was ≤ 2 cm, and resistant if it was >2 cm.

Kuk *et al.* (2003), described a rapid and reliable test to identify ALS-resistant monochoria (*Monochoria vaginalis*). The tests are seed germination in vivo and in vitro ALS activity, leaf, and greenhouse tests. In greenhouse tests, the R biotype was highly resistant to imazosulfuron and pyrazosulfuron-ethyl than the S biotype. Despite more reliable, greenhouse test have some disadvantages such as high cost, special infrastructure requirement and long term duration. In Petri-dish tests (seed germination test), the resistance level of the R biotype was 200-fold to imazosulfuron and 100-fold to pyrazosulfuron-ethyl than that of the S biotype. Petri-dishes test does not need as much infrastructure as a greenhouse test and can be finished in a shorter time. Leaf tests confirmed that leaf color of the R biotype was 1,600-fold to imazosulfuron and 300-fold to pyrazosulfuron- ethyl less affected, compared with that of the S biotype. This test takes approximately 6 days to complete. In vivo, the ALS test showed lower levels of resistance to both herbicides than did in vitro ALS test. All tests are able to distinguish R from the S biotype. The in vitro ALS test was selected as the best technique for future validation of resistance in *M. vaginalis* populations.

Burke *et al.* (2006), developed seedling bioassay to detect clethodim and fluazifop-P resistance in johnsongrass (*Sorghum halepense* (L.) Pers.). The test was based on differences in the coleoptile length of S and R seeds exposed to herbicides in Petri-dishes for five days. Bioassay concentrations of 0.09 mg/L clethodim and 0.18 mg/L fluazifop-P were chosen as discriminant based on rate responses of each biotype to increasing herbicide dose. Coleoptile length was measured and GR_{50} for the R and S seeds treated with clethodim were 462.5 and 24.8 mg/L, respectively.

R/S ratio was 18.7. The GR_{50} values for the R and S seeds were treated with fluazifop 618.7 and 17.5 mg/L, respectively, R/S ratio were 35.4.

Ballot *et al.* (2009), grown seeds of *Lolium rigidum* in the wells of ELISA plates with various glyphosate concentrations, measured the coleoptile length after 7 days and compared with means of pot test in the greenhouse. The test can be used to detect glyphosate resistance in other grass weeds. With some adaptation, it can be used to detect glyphosate resistance in dicotyledonous.

Cutulle *et al.* (2009), conducted screening tests in different conditions (Murashige and Skoog MS media, filter paper, hydroponics, and soil-based) to detect resistance to dithiopyr, prodiamine, and pendimethalin in annual bluegrass (*Poa annua* L.). All tests were able to identify resistance to prodiamine and pendimethalin. In hydroponics test, the amount of prodiamine required to inhibit growth by 50% was 26 times more than the control. In MS media, the amount of prodiamine required to inhibit by 50% growth was 80 times more than the control. Hydroponics has provided the most rapid (10 days) diagnosis of resistance.

Xu *et al.* (2010), conducted dose-response experiments in 9-cm diameter plastic Petri-dishes. Seeds of Flixweed (*Descurainia sophia* L.) were placed on two sheets of filter paper placed into the petri dish after immersing in 0.1% gibberellin for 12 h. ALS herbicide (Tribenuron-Methyl) was applied at different concentrations, untreated control was included. Petri dishes were placed in a growth chamber under adjusted condition. Shoot lengths were measured seven DAT. The method is regarded as the most rapid and simple way to screen resistant and susceptible biotypes.

Kaundun *et al.* (2011b), described a novel test to identify resistance to ALS and ACCase herbicides in grass weeds. The test was started by collecting seedlings from fields and transplanted onto agar medium amended different concentration of herbicides. The survived plant was recorded 10 days after transplanting and compared with S biotype. The test was able to detect resistance

and efficacy of herbicide in the growing season. The test was distinguished from the others as the Syngenta Resistance In-Season Quick test (RISQ).

Kaiser *et al.* (2013), mentioned a new quantitative herbicide-resistance test based on chlorophyll fluorescence imaging analysis of photosynthesis parameters. S and R of black-grass (*Alopecurus myosuroides*) were grown in multiwell plates containing agar amended with fenoxaprop-P-ethyl and mesosulfuron + iodosulfuron. He measured the maximum quantum efficiency of the PSII 3 h after transplanting and then every 24 h for 7 days. Data were compared with the greenhouse test. He confirmed that the chlorophyll fluorescence imaging correlated well with the greenhouse test and the test provided reliable data on herbicide tested in a shorter period and less space, compared with the greenhouse test.

Kaundun *et al.* (2014), described a simple, early-season test to detect glyphosate resistance in the grass and broadleaf weeds. He transplanted suspected R and S seedlings into agar medium with different rates of herbicide and the percentage of surviving plants was recorded after 14 days of transplanting. The test was validated using R and S biotypes of *Lolium*, *Eleusine*, *Conyza*, and *Amaranthus* species encompassing the main glyphosate resistance mechanisms. Greenhouse test and agar-based seedling tests made comparable resistance indices in dose-response and percentage survival at discriminating rates. Additionally, the test was suitable for other grass and broadleaf weeds that had evolved resistance to glyphosate. In addition, the method is simple, quick, cost-effective, and allows for evaluating glyphosate resistance-weeds in the growing season.

Zhang *et al.* (2016), measured the maximum quantum yield of PS II [Fv/Fm = (Fm –Fo)/Fm] at the 4–5 leaf stage to differentiate between R and S biotypes of barnyard grass (*Echinochloa* spp.) and confirmed by the whole-plant test based on ED_{50}. A PS II herbicide caused the fastest inhibition of Fv/Fm, compared with ACCase and ALS herbicide treatment. The required period for distinguishing between R and S was 64 h after PS II herbicide treatment, 168 and 192 h after ACCase and ALS herbicide, respectively. Chlorophyll fluorescence test provided a

reliable method of herbicide resistance in barnyard grass, time savings and suitable measurement in field environments compared with whole-plant pot test.

Brosnan *et al.* (2017), developed an agar-based diagnostic test for agronomic weeds to evaluate herbicide resistance in annual bluegrass to ALS, EPSPS, and PSII inhibiting herbicides. Seedlings were transplanted into autoclavable polycarbonate plant culture boxes filled with agar amended with a murashigee-Skoog medium and a range of herbicide concentrations. Mortality in agar was assessed 7-10 DAT and compared to the responses observed after treating individual plants in the greenhouse with normal recommended field dose.

Linn *et al.* (2018), studied on the feasibility of a chlorophyll fluorescence agar-based test on herbicide resistance in *Apera spica-venti* L. Seedlings of R and S biotypes were transplanted into agar medium containing pinoxaden and pyroxsulam. 48 and 72 h after the transplant, chlorophyll fluorescence and the maximum quantum efficiency of photosystem II (Fv/Fm) was determined, respectively. Each experiment repetition exhibited different sensitivities of the biotypes for both herbicides.

2.3.2. Greenhouse Test for Detecting Herbicide Resistance

The greenhouse test (also known as whole-plant pot or classical assay) start by sowing seeds in pots and apply either pre-emergence (PRE) or post-emergence (POST) herbicides at the recommended growth stage using BBCH scale (Hess *et al.*, 1997). Field soil must be used to obtain accurate PRE herbicide activity, whereas commercial potting soils can be used for POST emergence (Burgos *et al.*, 2013). To evaluate the efficacy of herbicides, different data should be collected, for example, the number of live plants, injury level, and shoot biomass or dry weight measurement after 7-14 days after treatment (DAT) for the fast-acting herbicide, then again at 28 to 30 DAT to observe any regrowth. For slow-acting herbicides (e.g. glyphosate) can be evaluated within 3-4 week after treatment (WAT) (Burgos, 2015).

2.3.3. Molecular-Based Tests for Detecting Herbicide Resistance

Many resistance cases have been detected at the molecular level. DNA-based tests have the potential to provide precise and rapid identification of resistance (Corbett and Tardif, 2006). Kaundun *et al.* (2011a) developed a novel method to detect resistance at the level of the DNA of mutations, analysis of known mutations using dCAPs assay (Massa *et al.*, 2011; Pan *et al.*, 2016), real-time quantitative PCR (Kaundun *et al.*, 2006), and Pyro-sequencing (Petersen *et al.*, 2010; Riggins *et al.*, 2010) have been used for detecting herbicide resistance.

Siminszky *et al.* (2005), investigate the possibility of using denaturing high-performance liquid chromatography (DHPLC), a newly developed technique for mutation analysis, for the finding of three ALS mutations, AlaI22Thr, Leu574Trp, and Ser653Thr, which confer herbicide resistance. The mutated variants of the ALS gene were isolated from resistant biotypes of smooth pigweed and Powell amaranth using polymerase chain reaction (PCR) and hybridized with wild-type and exposed to DHPLC. The sensitivity of the technique was strongly reliant on the temperature profile of the examined DNA fragment. The process is inexpensive, fast, and requires little sample preparation. These advantageous features, DHPLC can be used as an alternative to other normally used mutation detection techniques.

Délye and Boucansaud (2008), used the derived cleaved amplified polymorphic sequence (dCAPs) method to detect a change ALS codons A122, P197, A205, W574 and S653 in *Alopecurus myosuroides.* These tests identified W574L or P197T, in most plants examined from a field where ALS herbicide unsuccessful after 3 years of applications. These tests would be very suitable in proactive resistance confirmation method.

Lamego *et al.* (2009), described at the molecular basis of resistance in greater beggar-ticks (*Bidens subalternans* DC.), ALS gene was sequenced and compared between S and R biotypes. Analysis of the nucleotide and amino acid

sequences between the biotypes indicated that a single point mutation, G–T, in one ALS isoform from the R biotype resulted in an amino acid substitution, Trp574Leu.

Délye *et al.* (2011b), developed a dCAPs technique to detect amino acid substitutes at 7 ACCase codons (1781, 1999, 2027, 2041, 2078, 2088 and 2096) and at 2 ALS codons (197 and 574). The nine tests were effectively used to genotype ACCase and ALS in 39 grass weed species. Their flexible design allows an easy finding of new changes at the targeted codons. These tests are tools to easily identify resistance caused by modification(s) of ACCase or ALS herbicide in weed species.

Alarcón-reverte *et al.* (2013), described a SNaPshot test to screen up to 10 mutations from TSR related codons in a single reaction. The test was complex, successfully used for the genotyping of nine variable nucleotide positions in the CT domain of the ACCase gene from *Lolium multiflorum*. The advantage of the test was able to distinguish between different nucleotide changes at a single locus.

Yuan *et al.* (2015), at the molecular level, confirmed a Trp1999-Ser mutation in the R biotype of *Pseudosclerochloa kengiana*. Positively determined wild type Trp and mutant Ser alleles at ACCase position 1999 in two dCAPS markers. Individual plants analyzed showed heterozygous mutants. The analysis of plant genotype and phenotype showed that wild type plants were killed after treatment with any one of the ACCase herbicides.

Darmency and Uludag (2018), used quantitative genetic tools to assess whether a low dose of herbicide drive selection on standing genetic difference in populations evolved NTSR. The heritability index (H) provides a measure of the possible to develop NTSR and can be simply calculated from greenhouse experiments. This measure and the related experimental designs are discussed with two applied examples on Avena spp. (*A. fatua* and *A. sterilis*). The H values ranged from 0.24 to 0.73, which means that selection for NTSR is highly probable in cases with high H value. These tools are important to estimate the number of genes

involved and the potential speed of evolution without increasing experimental cost and time.

3. MATERIAL AND METHODS

3.1. Material

The main material of the study consists of sterile wild oat seeds; ACCase-inhibiting herbicides (clodinafop-propargyl+cloquintocet-mexyl and pinoxaden+ cloquintocet-mexyl), ALS-inhibiting herbicides (mesosulfuron-methyl+ iodosulfuron-methyl-sodium and pyroxsulam+cloquintocet-mexyl), pots, Petri-dishes, plant agar, rechargeable backpack sprayer (MATABI) and other laboratory equipment's are used. Some of these materials are given detailed below.

3.1.1. Sterile Wild Oat (*Avena sterilis* L.) Description and Distribution

Sterile wild oat is a major annual grass weed in general appearance resembles with wheat and cultivated oats. There are three sub-species of sterile wild oat, *A. sterilis* subsp. *sterilis, A. sterilis* subsp. *ludoviciana* and *A. sterilis* subsp. *trichophylla*. *A. sterilis* is mainly self-pollinating, prolific seed producers, for instance, one plant can produce 225-400 seeds, and a high infestation can produce 20,000 seeds/m^2 (Fernandez-Moreno *et al.*, 2016). 80% of sterile wild oat seed is lost before wheat harvest (Tidemann *et al.*, 2016), it has primary and secondary seed dormancy (Beckie *et al.*, 2012a) allowing it to live in a seed-bank for 4 to 5 years (Van Acker, 2009). Sterile wild oat classified as follows:

Kingdom: Plantae (Plants)
Sub-kingdom: Tracheobionta (Vascular plants)
Super-division: Spermatophyta (Seed plants)
Division: Magnoliophyta (Flowering plants)
Class: Liliopsida (Monocotyledons)
Sub-class: Commelinidae
Order: Poales
Family: Poaceae/Gramineae
Genus: Avena
Species: *sterilis*

Synonyms of *Avena sterilis* L.:

Avena fatua var. *sterilis* (L.) Mérat
Avena macrocarpa Moench, nom. superfl.
Avena nutans St.-Lag., nom. superfl.
Avena sativa subsp. *sterilis* (L.) De Wet
Avena sativa var. *sterilis* (L.) Fiori

Seeds of *A. sterilis* are varied in color from black to yellow and may have gold-brown hairs (Figure 3.1) and have the high germination ability; it can germinate under a wide range of temperatures 5-30°C (Fernandez-Quinantilla *et al.*, 1990; McGillion *et al.*, 2006; Tünk, 2018). Seedlings of *A. sterilis* usually twist in an anticlockwise direction as opposed to the wheat crop. Mature plants are up to 120 cm tall, without branching and rooting at nodes. They have some hairs on their leaves, a large ligule (2 mm), and no auricles. The spikelet is hard and does not break easily, seeds remain undispersed at maturity. Glumes are equal, 30-50 mm long (Schmid and Stace, 1998; Moss, 2015).

A. sterilis is widely distributed (Figure 3.2), is being found in: parts of the USA; Central and South America; southern, eastern and northern Africa; central and southeastern Europe; the Middle East; the Indian subcontinent; predominate in all Mediterranean region which include Mediterranean sea countries, southern Chile, California, Cape town of South Africa and southern Australia (Travlos and Giannopolitis, 2010; Gonzalez-Andujar *et al.*, 2010). Turkey has been considered as a center of iso-enzyme diversity in *A. sterilis* (Phillips *et al.*, 1993). In Turkey, it is found in part of the Mediterranean region (Adana, Mersin, and Isparta), southeast of Anatolia (Şanlıurfa, Diyarbakır, and Mardin), it’s also found in Istanbul, Izmir, and Muğla (Figure 3.3), and it’s become more common in Çukurova with it's in 84.85% frequency in surveying fields (Uygur, 1985).

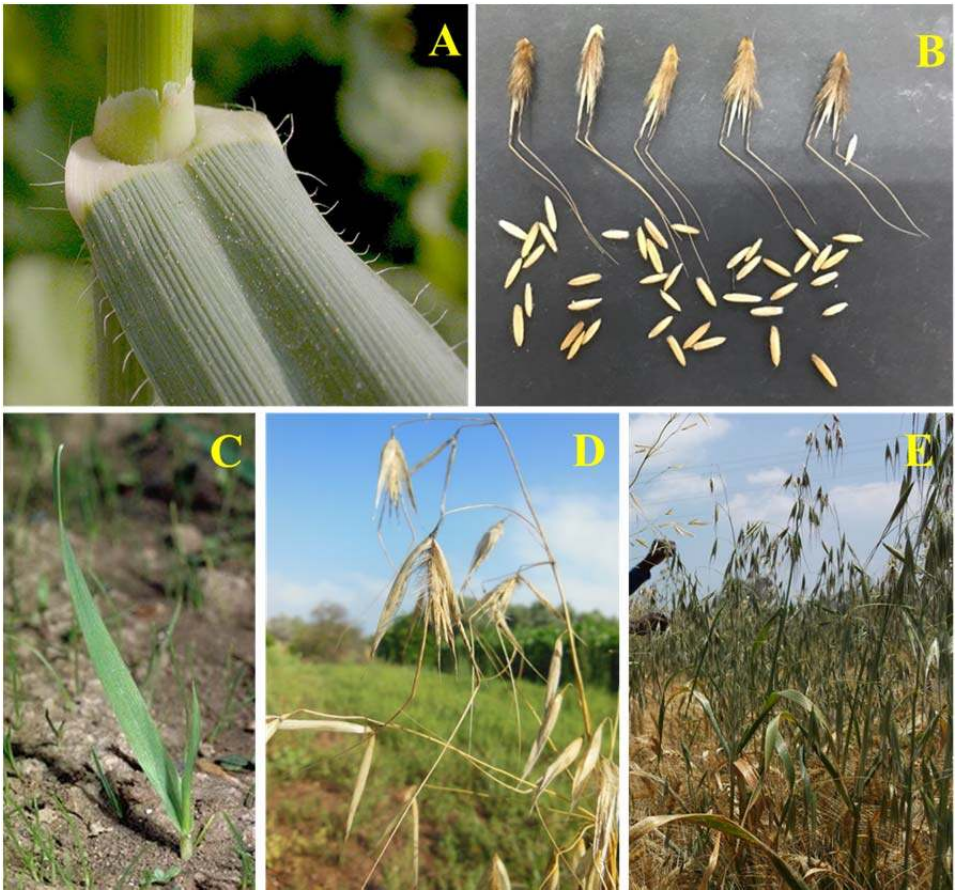

Figure 3.1. Morphological appearance of sterile wild oat (*Avena sterilis* L.), ligula (A), spikelet's, florets and seed (B), seedling (C) and whole plant with flower head (D, E). From Çukurova University Weed Science Lab.

Figure 3.2. Worldwide distribution of sterile wild oat (*Avena sterilis* L.) (CABI, 2019).

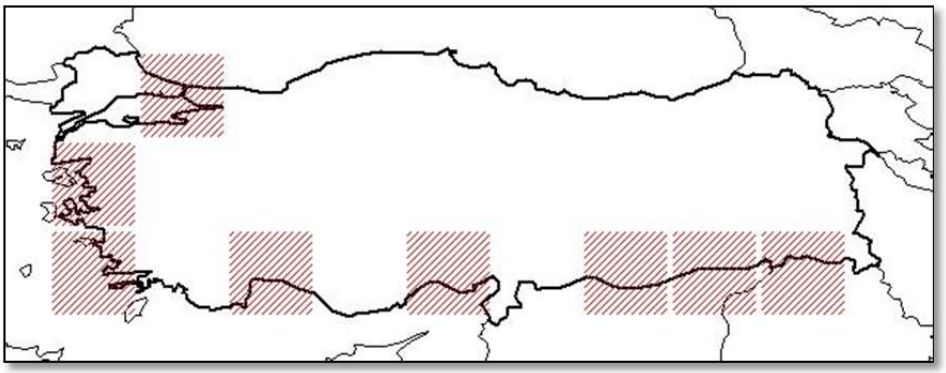

Figure 3.3. Distribution of sterile wild oat (*Avena sterilis* L.) in Turkey (TÜBİVES, 2019).

3.1.2. ACCase-Inhibiting Herbicides (HRAC Group A, WSSA Group 1)

Acetyl-CoA carboxylase (ACCase)-inhibiting herbicides were discovered in 1978 and commercialized in the early of the 1980s (Beckie and Tardif, 2012; Martins *et al.*, 2014). ACCase herbicide catalyzes the adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA to produce malonyl-CoA (figure 3.4), which was the first committed enzyme in fatty acid biosynthesis (Guo *et al.*,

2015; Dayan *et al.*, 2015; Guo *et al.*, 2017; Du *et al.*, 2019). There are three chemical families of ACCase-inhibiting herbicides: aryloxyphenoxypropionate (AOPP or FOPs), cyclohexanedione (CHD or DIMs) and phenylpyrazoline (DEN) (Wenger *et al.*, 2012; Zhao *et al.*, 2019). ACCase herbicides have been widely used as a selective, post-emergence to control grass weed species such as *Avena* spp. in broad-leaved crops such as oilseed rape and legumes (De Prado and Franco, 2004; DÉLye *et al.*, 2011a; Kukorelli *et al.*, 2013). It's absorbed through the foliage and translocate in the phloem to the growing point, where they inhibit meristematic activity. Symptoms of ACCase herbicides appear several days after treatment and its severity depending on the crop, the rate of application, and growth stage. Symptoms, in general, are chlorosis, necrosis and finally the death of plant tissue (Ball *et al.*, 2007; Kaundun, 2014).

3.1.2.1. Clodinafop-Propargyl + Cloquintocet-Mexyl

Clodinafop-propargyl (aryloxyphenoxypropionate) a systemic post-emergence and foliar-absorbed herbicide (Abbaspoor and Streibig, 2005). It controls annual and perennial grasses, e.g., Canarygrass, *Phalaris paradoxa* L. (Collavo *et al.*, 2011); wild oat, *Avena sterilis* L. (Papapanagiotou *et al.*, 2012); black-grass, *Alopecurus myosuroides* Huds (Délye *et al.*, 2005; Petit *et al.*, 2010a) and rigid ryegrass, *Lolium perenne* L (Scarabel *et al.*, 2011; Yu *et al.*, 2007).

Table 3.1. Chemical and physical properties of clodinafop-propargyl (Anonymous, 2019b)

Common name	clodinafop-propargyl
Chemical name(IUPAC)	prop-2-ynyl (R)-2-phenoxy] propionate
Chemical formula	$C_{17}H_{13}ClFNO_4$
Molecular weight	349.742 g/mol
LD_{50} value	1829 mg/kg
Melting point	59.5^{o}C

3.1.2.2. Pinoxaden + Cloquintocet-Mexyl

Pinoxaden (phenylpyrazoline) herbicide introduced in 2006, used as post-emergent against a wide range of grass weed species such as *Alopecurus myosuroides*, *Apera spica-venti*, *Avena* spp., *Lolium* spp., *Phalaris* spp. and *Setaria* spp. (Delye, 2005; Yu *et al.*, 2010).

Table 3.2. Chemical and physical properties of pinoxaden (Anonymous, 2019b)

Common name	pinoxaden, abbreviation PXD
Chemical name (IUPAC)	8-(2,6-diethyl-4-methylphenyl)-1,2,4,5-tetrahydro-7-oxo-7 *H*-pyrazolo [1,4,5]oxadiazepin-9-yl 2,2-dimethylpropanoate
Chemical formula	$C_{23}H_{32}N_2O_4$
Molecular weight	400.519 g/mol
LD_{50} value	> 5000 mg/kg
Melting point	120.5-121.6°C

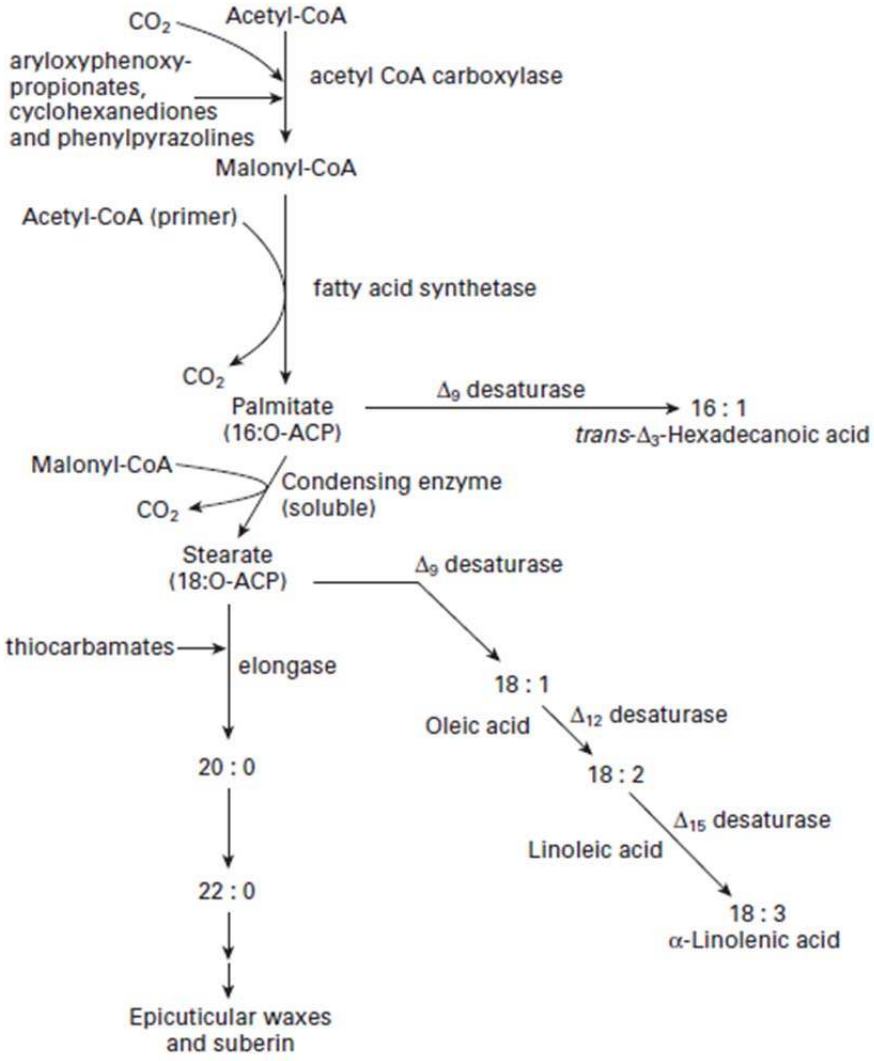

Figure 3.4. Fatty acid biosynthesis in plants. ACP=acyl carrier protein (Cobb and Reade, 2010).

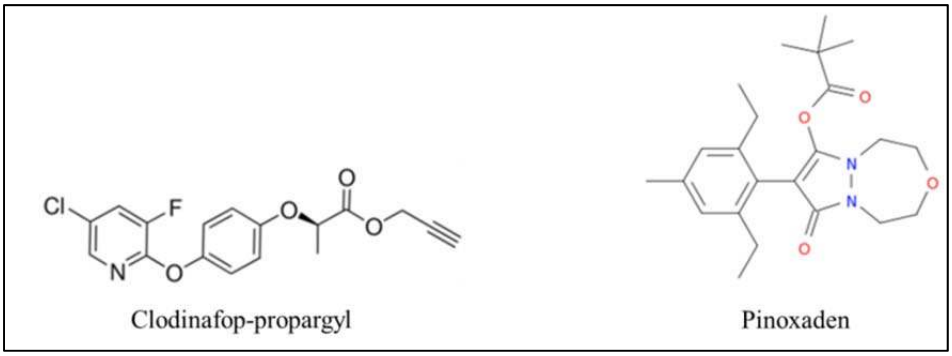

Figure 3.5. Chemical structure of clodinafop-propargyl and pinoxaden (Anonymous, 2019b).

3.1.3. ALS-Inhibiting Herbicides (HRAC Group B, WSSA Group 2)

At the beginning of the 1980s, acetolactate synthase (ALS, also known as acetohydroxyacid, AHAS) was discovered and launched into the market for the control of broad-leaved and grass weeds (Tranel and Wright, 2002; Kaundun *et al.*, 2012; Li *et al.*, 2017a). Currently, there are 54 registered ALS inhibitor herbicides, and they account for one-sixth (54/302) of all registered herbicides (Heap, 2014). ALS is the first enzyme in the pathway to the branched-chain amino acid biosynthesis (Valine, Leucine, and Isoleucine) (figure 3.6), which catalysis two distinct reactions in plants (Duggleby and Pang, 2000; Tardif *et al.*, 2006; Duggleby *et al.*, 2008; Babineau *et al.*, 2017). ALS herbicide comprises the largest site-of-action across five families; sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyl-thiobenzoates (PTBs) and sulfonyl-aminocarbonyl-triazolinones (SCTs) (Yu and Powles, 2014). ALS herbicides have been widely used among farmers globally due to their broad-spectrum (Moss, 1991; Heap, 1997) high selectivity, high efficiency at low rates and it has low mammalian toxicity (Saari *et al.*, 1994; Shen *et al.*, 2009; Choe *et al.*, 2015) and less environmentally hazardous (Vercellino *et al.*, 2018). ALS herbicide, absorbed by roots and foliage translocate by xylem and phloem to the site of action at the growing points. The symptoms caused by ALS herbicides are not apparent until

several days after treatment. Symptoms arising from ALS herbicide are chlorosis, yellowing of new growth, purpling of veins, stunting, inhibition of lateral root growth giving a bottle-brush appearance, wilting, and turning red due to the accumulation of stress-induced anthocyanin's (Loper *et al.*, 2002; Corbett and Tardif, 2006; Anonymous, 2019a).

3.1.3.1. Mesosulfuron-Methyl + Iodosulfuron-Methyl-Sodium

Mesosulfuron-methyl is a selective sulfonylurea herbicide, commercialized in 2001 (Hacker *et al.*, 2002), and registered for use on wheat in 2004 (Chandi *et al.*, 2011). Applied post-emergence (POST) alone or in mixture with iodosulfuron to control grass weeds, e.g., black-grass, wild oats and Italian ryegrass (Bailey and Wilson, 2003; Bailey *et al.*, 2003; Crooks *et al.*, 2003; King, 2007). It becomes the most important herbicide for controlling ACCase herbicide resistant-weed (Bi *et al.*, 2013). It is well-known for its low animal toxicity, high efficacy, a broad spectrum and used worldwide (Powles and Yu, 2010).

Table 3.3. Chemical and physical properties of mesosulfuron-methyl (Anonymous, 2019b)

Common name	Mesosulfuron-methyl
Chemical name(IUPAC)	methyl 2-[(4,6-dimethoxypyrimidin-2-yl) carbamoyl sulfamoyl]-4-(methanesulfonamidomethyl) benzoate
Chemical formula	$C_{17}H_{21}N_5O_9S_2$
Molecular weight	503.501 g/mol
LD_{50} value	>5000 mg/kg
Melting point	195.4^{o}C

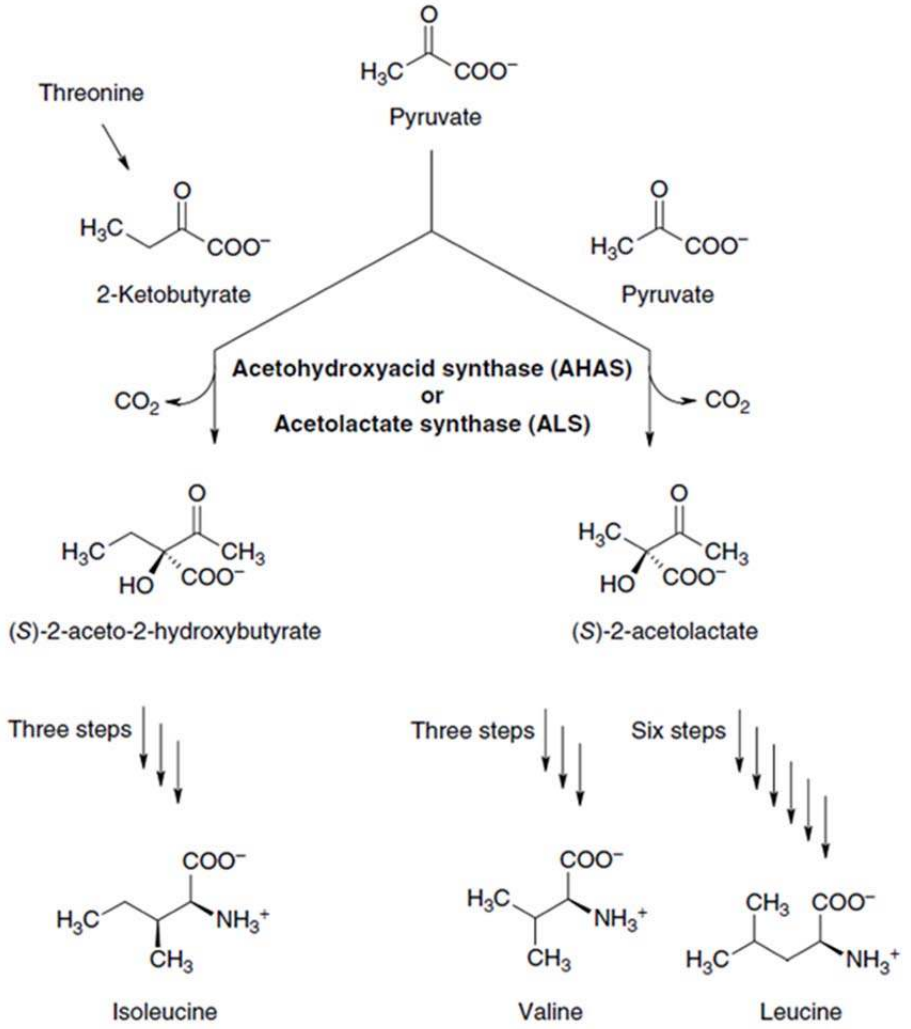

Figure 3.6. Branched-chain amino acid biosynthetic pathway (Thompson, 2007).

3.1.3.2. Pyroxsulam + Cloquintocet-Mexyl

Pyroxsulam is the latest triazolopyrimidine sulfonamide manufactured and commercialized in 2006-2007 (EPA, 2008; Gonzalez *et al.*, 2008; Wells, 2008). Pyroxsulam provides selective post-emergent control, registered worldwide to control annual grass and broadleaf weeds in certain cereals (Layton and Kellogg, 2014; Zobiole *et al.*, 2018).

Table 3.4. Chemical and physical properties of pyroxsulam (Anonymous, 2019b)

Common name	Pyroxsulam
Chemical name(IUPAC)	N-(5,7-dimethoxy[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)-2-methoxy-4-(trifluoromethyl)-3-pyridinesulfonamide
Chemical formula	$C_{14}H_{13}F_3N_6O_5S$
Molecular weight	434.35 g/mol
LD_{50} value	>2000 mg/kg
Melting point	208.3°C

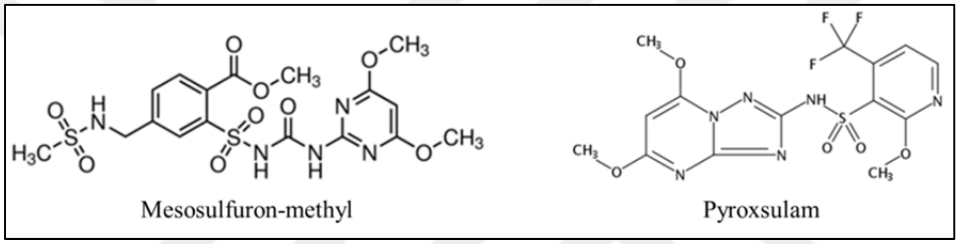

Figure 3.7. Chemical structure of mesosulfuron-methyl and pyroxsulam (Anonymous, 2019b).

3.2. Method

3.2.1. Seed Samples Collection of *Avena sterilis* L. in the Fields

In summer (May-June) 2016, seeds of *A. sterilis* populations previously reported as resistant to ACCase and ALS inhibiting herbicides were collected from a wheat field in the province of Kozan (Adana, Turkey [37°28′14.52″ N, 35°45′59.82″ E]). The large sample size of 20,000 seeds of the resistant *A. sterilis* population was collected randomly from multiple plants from different areas within the single field (Figure 3.8) (Falk *et al.*, 2005; Abdurruhman and Uygur, 2018c). The susceptible population was sampled from the borders of the same fields (> 4 m from the field sites) that had never been treated with herbicides (Burgos *et al.*, 2013). Seeds were then cleaned, air-dried and stored in glass Petri-dishes and kept in the refrigerator at 4°C to break seed dormancy (Kuk *et al.*, 2003; Bewley *et al.*, 2012; Uygur *et al.*, 2013; Feng *et al.*, 2016).

Figure 3.8. Wheat-field infested by herbicide-resistant sterile wild oat where seed samples were collected.

3.2.2. Quick-Test for Detecting Herbicide Resistance in *Avena sterilis* L.

3.2.2.1. Agar-Based Seed Germination Test

The experiment was conducted by modifying previous studies of Abdurruhman *et al.* (2018). Plant agar (Pl00l, Duchefa) 0.9% *w/v* was liquefied in a microwave and allowed to cool around 35-40°C before the commercial herbicide solutions were added at the targeted concentrations. The concentrations of 0, 1.2, 2.4, 4.8, 9.6, 19.2 and 38.4 µM for clodinafop-propargyl + cloquintocet mexyl; 0, 0.225, 0.45, 0.9, 1.8, 3.6 and 7.2 µM for pinoxaden + cloquintocet-mexyl; 0, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 µM for mesosulfuron-methyl + iodosulfuron-mexyl; and 0, 0.37, 0.75, 1.5, 3, 6 and 12 µM for pyroxsulam + cloquintocet-mexyl were used.

A total of 20 ml agar medium was poured into 9-cm diameter glass Petri-dishes (Figure 3.9). Eventually, 10 seeds were placed in each Petri-dish and the dishes were covered and kept in a refrigerator at 10°C. Petri-dishes were arranged in a complete randomized design with six replicates of each concentration and the entire experiment was repeated twice. The percentage of seed germination means of the coleoptile and hypocotyl (Figure 3.10) lengths were measured 15 days after incubation.

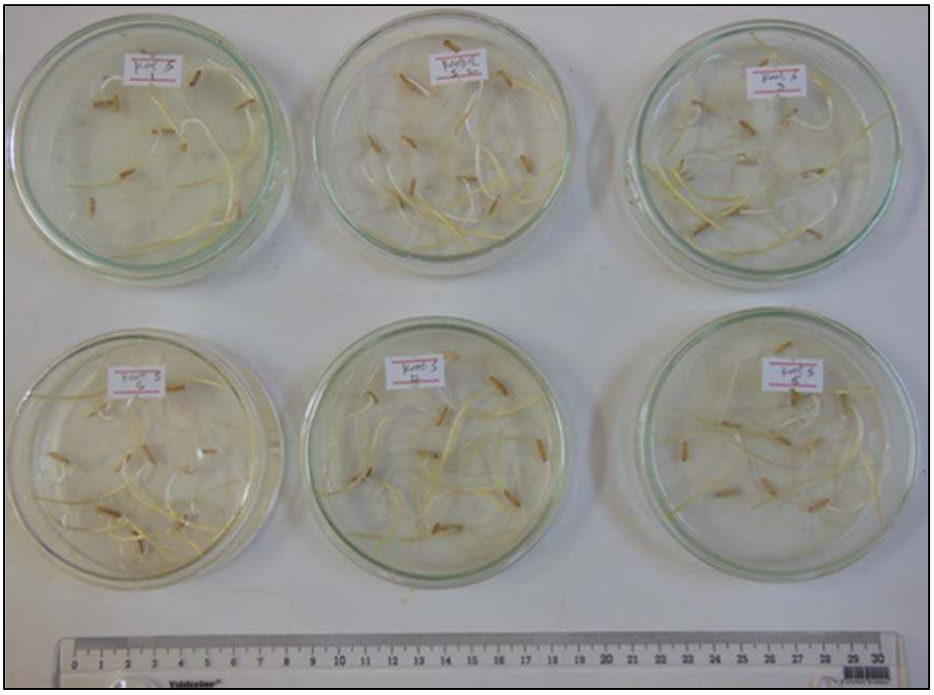

Figure 3.9. Seeds of sterile wild oat (*Avena sterilis* L.) germinated in Petri-dishes containing agar medium, after 15 days of incubation.

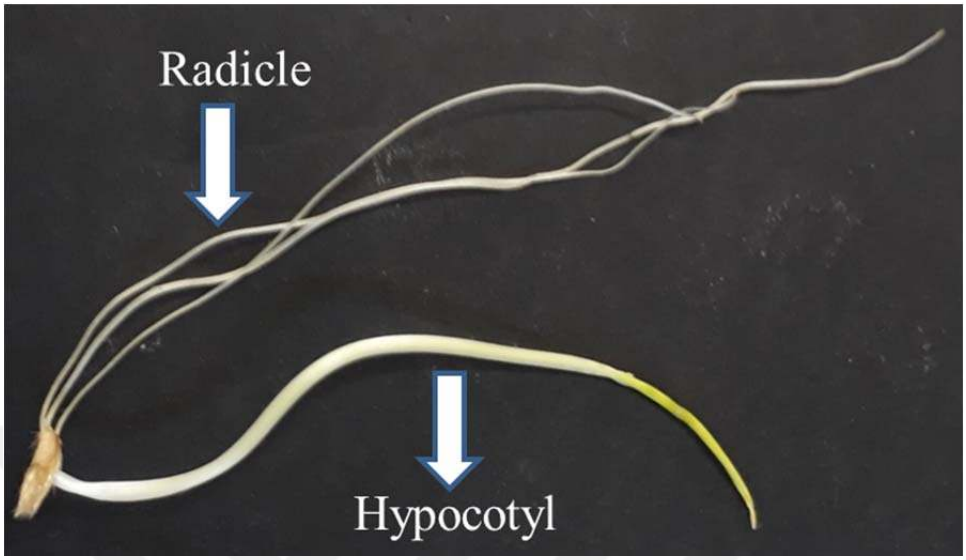

Figure 3.10. Coleoptile and hypocotyl of sterile wild oat (*Avena sterilis* L.).

3.2.2.2. Agar-Based Seedling Test

The seedling assay was carried out in the greenhouse in Flakkebjerg Research Center, Aarhus University, Denmark, during the winter, 2018 (November to December). Seeds from the R and S populations were grown in seedling trays in the greenhouse adjusted at 60% relative humidity, 12/6^{o}C, 10/14 h day/night. The agar was prepared similar to that mentioned above in the seed germination test. Two herbicides were used in this test. 100g L^{-1} clodinafop-propargyl + 25g L^{-1} cloquintocet mexyl, at rates of 0, 0.65, 0.135, 0.27, 0.54, and 1.08 mg L^{-1}; 10g L^{-1} mesosulfuron-methyl + 2g L^{-1} iodosulfuron-methyl-Na + 30g L^{-1} mefenpyr-diethyl, at rates of 0, 0.3, 0.6, 1.2, 2.4 and 4.8 mg L^{-1}. The amount of 100 ml agar medium containing herbicide solution was poured into 12 x12 cm diameter plastic Petri-dishes (Figure 3.11). Agar media was settled in the room at the 10^{o}C for 24 hours. Seedlings of sterile wild oat at 2-3 leaf stage (BBCH 12-13) (Hess *et al.*, 1997) were collected from trays and washed using tap water to remove soil from the root (Kaundun *et al.*, 2011b). The growing points of seedlings (leaf and root) were

excised using scissors. The root of the seedling was carefully placed into the agar medium using forceps and the dishes were covered with a lid and placed in the greenhouse. The experiment was arranged in a completely randomized design, five seedlings per Petri-dish with three replicates. The estimation of herbicide efficacy consisted of visual rating for survival seedling growth of new root and shoot 15 days after transplanting (DAT) (Kaundun *et al.*, 2014).

Figure 3.11. Transplanted seedlings of sterile wild oat (*Avena sterilis* L.) in Petri-dishes containing agar medium.

3.2.2.3. Filter Paper-Based Seed Germination Test

The experiment was carried out in a growth cabinet according to Uygur *et al.* (2013). Five seeds each from R and S populations of *A. sterilis* were germinated in 4.5-cm in diameter Petri-dish on the top of two layers of filter paper (Figure 3.12). Water was subsequently added when needed. To maintain the humidity inside the Petri-dishes, an amount of 30g of fine sand was added into each Petri-dish after the seed germination started. At the two-leaf stage, seedlings were thinned to two plants per Petri-dish. Afterward, the Petri-dishes were placed in the glasshouse at a temperature of 20/8^{o}C, 10/14 h day/night, 30% relative humidity (RH) and a light intensity of 250µmol quanta m^{-2} s^{-1} of photon flux. Herbicides and their application

rates are given in Table 3.5. Six doses (0, N/4, N/2, N=recommended field dose, 2N, 4N and 8N) for each herbicide and untreated control are used. To improve herbicide performance, Biopowder* (1000 ml ha^{-1}) and Dassoil 26-2N (500 ml ha^{-1}) was added to mesosulfuron-methyl+iodosulfuron-methyl-sodium and Pyroxsulam+cloquintocet-mexyl spray solutions, respectively. Herbicides were applied outdoors at the recommended growth stage (BBCH 13-14) arranged in an area of 10 m^2. Herbicide application was done by a rechargeable backpack sprayer (MATABI) equipped with Tee-Jet nozzles with application volume of 250 L ha^{-1} at 303.975 kPa and boom height of 50-cm from the weed. Herbicide doses were applied from the lowest to the highest concentrations, then the pots were placed in separate trays to avoid cross-contamination of different herbicide concentrations. The experiment was carried out in a completely randomized design with six replicates of each herbicide rate and the whole experiment was repeated four times. The levels of injury and plant height were recorded a day before treatment and at 1, 3, 5, 7, 14, 21, and 28 DAT (Beckie *et al.*, 2004).

Figure 3.12. Filter paper-based seed germination test in daylight cabin.

3.2.2.4. Greenhouse Test for Detecting Herbicide Resistance in *Avena sterilis* L.

The experiment was conducted according to the herbicide resistance action committee (HRAC) rules. A study was done in a polyethylene-covered greenhouse

at the experimental farm of Plant Protection Department of Çukurova University, during the winter season (November-March, 2016 and 2017). Five seeds from R and S populations were sown in pots (10 x 15 cm) containing a potting mix (field soil, sand, and peat) in a 1:1:1 ratio. Pots were placed in the greenhouse (20/8^{o}C, 14/10 h day/night and 25% relative humidity) and subsequently irrigated. After germination, seedlings were irrigated every 48 to 72 hours to avoid moisture stress. Later on, *A. sterilis* seedlings were thinned into two plants per pot. One week later, herbicides were applied and the spray condition was similar to that mentioned above in the Petri-dish assay. The experiment was conducted in a completely randomized design (CRD) with six replicates for each concentration (Figure 3.13). To assess the herbicide efficacy, plant height was recorded and levels of herbicide injury were estimated at 7, 14, 21 and 28 days, relative to untreated control (Beckie *et al.*, 2004). Then, at 28th day of the experiment, plants were harvested above ground, dried in an oven at 65^{o}C for 72 h (Ahmad-Hamdani *et al.*, 2012) and dry weights were obtained. To verify the results, the entire experiment was repeated four times (Maneechote *et al.*, 2005; Burgos *et al.*, 2013).

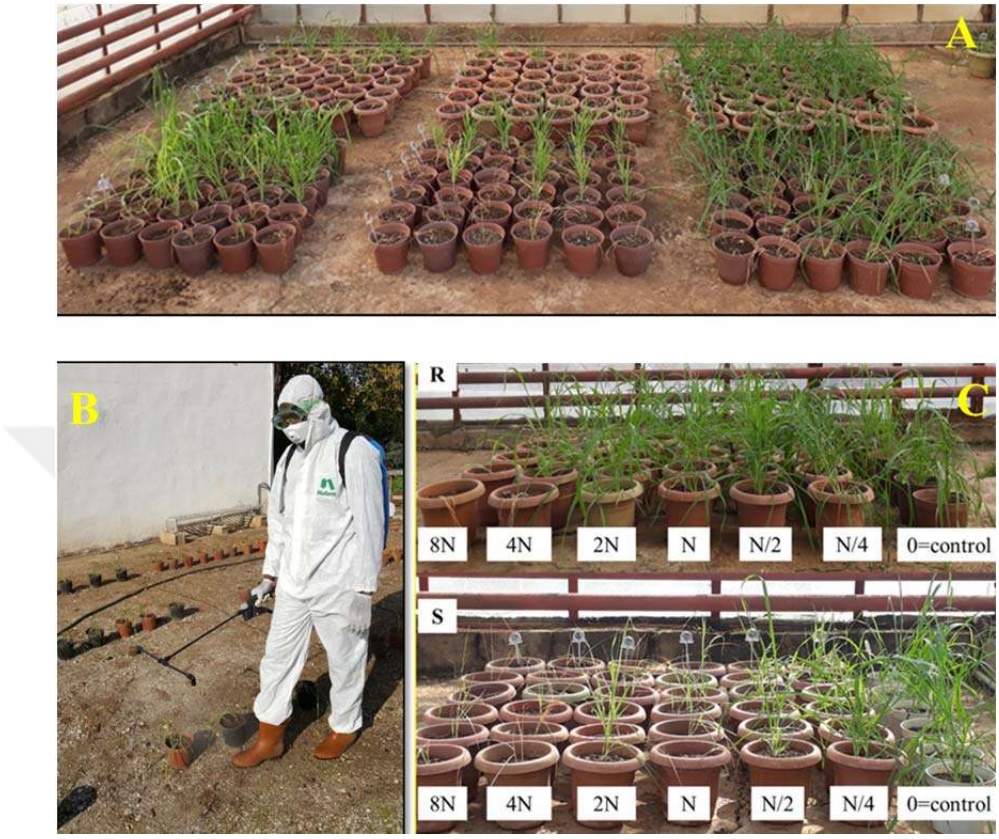

Figure 3.13. The layout of the greenhouse test (A), herbicide application (B) and resistant and susceptible sterile wild oat (*Avena sterilis* L.) populations plant treated with different herbicide at doses (C).

Table 3.5. Commercial formulation herbicide doses in Petri-dish and greenhouse test

Common name	Doses (g a.i h^{-1})						
	0	N/4	N/2	N	2N	4N	8N
Clodinafop-propargyl + cloquintocet-mexyl	0	12	24	48	96	192	384
Pinoxaden+cloquintocet-mexyl	0	10.12	20.2	40.5	81	162	324
Mesosulfuron-methyl+ iodosulfuron-methyl -sodium	0	2.25	4. 5	9	18	36	72
Pyroxsulam+cloquintocet-mexyl	0	4.68	9.37	18.7	37.5	75	150

3.2.2.5. Molecular Test for Detecting Herbicide Resistance in *Avena sterilis* L.

Plant materials for ALS and ACCase analysis from R and S populations were harvested above ground from plants survived the recommended herbicide dose in the four weeks after treatment (Yu *et al.*, 2008; Yu *et al.*, 2012). The samples were kept in plastic bags containing ice and stored at -20^{o}C. Genomic DNA extracted from R and S populations through CTAB (cetyltrimethylammonium bromide) protocol described by Doyle and Doyle (1987), Rychlik *et al.* (1990) and Delye *et al.* (2015). Total DNA was extracted in the following steps:

1. 100 ml of CTAB isolation buffer (10 ml 1M Tris-HCl pH 8, 28 ml 5M NaCl, 4 ml 0.5M EDTA, 0.2% 2-mercaptoethanol, 2g CTAB, and 40 ml deionized water) was dissolved by heating at 60^{o}C in a water bath and brought to 100 ml with deionized water.
2. 1 gram of plant material was taken from fresh tissue and placed in the extraction bag.
3. 4 ml CTAB buffer was added in the extraction bag and grounded by pestles.
4. 600 µl from the grounded sample was poured into 1.5 Eppendorf tubes and incubated at 65 ^{o}C for 1 hour.
5. Samples were centrifuged at 13,500 rpm for 5 minutes.
6. 600 µl of chloroform-isoamyl alcohol (24:1) was added, mixed by inversion and centrifuged at 13,500 rpm for 5 minutes.
7. The upper aqueous layer (500 µl) was transferred into 1.5 ml Eppendorf tube; an equal volume of isopropanol was added, vortexed, and centrifuged at 13,500 rpm for 10 minutes.
8. The supernatant was discarded, 250 µl of 80% ethanol was added and centrifuged at 13,500 rpm for 2 minutes.
9. The supernatant was discarded, 250 µl of 95% ethanol was added and centrifuged at 13,500 rpm for 2 minutes.

10. The supernatant was discarded and the pellet was completely dried at room temperature.
11. The dried DNA pellet was re-suspended in 100 µl of water or TE buffer and stored at -80 ^{o}C prior to PCR analysis.

Four DNA samples were extracted using the rapid protocol described by Delye *et al.* (2002a), where 1 cm plant tissue was cut, placed into a 0.5-ml microcentrifuge tube containing 200 µl of CTAB extraction buffer. Tubes were placed in water for 6 min at 95^{o}C, transferred into ice for 10 min and vortexes for the 20s. The primers used in the study are listed in Table 3.6.

A Polymerase chain reaction (PCR) was conducted using a Bio-Rad Thermocycler (Applied Biosystems) with the following reaction: 10 ng of genomic DNA, 6.25 µl DNA master Mix 2x, 2 µl of each primer (10 µm), 5 µl of 10x EasyTaq Buffer, 4 µl of dNTPs, and 0.5 µl of EasyTaq DNA polymerase, 1.5 mM MgCl, 50 Mm KCL, 10 Mm Tris-HCL (PH 8.3) added with ddH_2O to the final volume of 50 µl. The PCR was run for an initial denaturation of 94^{o}C for 1 min; followed by 35 cycles of 94^{o}C for the 40s, 58^{o}C for 1 min, 72^{o}C for 1 min; and a final step at 72^{o}C for 5 min used for ALS-inhibiting herbicides. In ACCase-inhibiting herbicide the initial denaturation step was 94^{o}C of 4 min followed by 35 cycles of the 30s at 94^{o}C, 30s at 62^{o}C, 30s at 72^{o}C and final step for 5 min at 72^{o}C was also included. The PCR product was visualized on 5% agarose gel with 2,000bp markers to confirm amplification size and sequenced.

Table 3.6. Primers sequences used for amplification of ACCase and ALS gene in *Avena sterilis* from genomic DNA

Primers	Sequence (5'-3')	Annealing temperature	size (bp)	References
ALS-F	AAGGGCGCGCGACATCCT	58	430	Marshall and Moss (2008)
ALS-R	ATCTGCTGCTTGGATGTCCTT			
ACCase-F	GCGTGCTGCTGGGCTCAAT	62	600	Gherekhloo *et al.* (2012)
ACCase-R	CCAGTTAAGATAATGGGCTGGTC			

3.2.3. Data Analysis

Data of shoot dry weight, plant height, coleoptile length, and hypocotyl length were converted to the percentage of the untreated control. Dose-response curves were estimated for each experiment separately. Data were subjected to non-linear regression analysis using four and three-parameter log-logistic model, equation 1 and 2 (Kudsk *et al.*, 1995; Seefeldt *et al.*, 1995) in the R statistical software (R development team, https://www.r-project.org) with add-on package *drc* (Ritz and Streibig, 2005; Knezevic *et al.*, 2007; Ritz *et al.*, 2015). The log-logistic model with three or four parameters (equation 1 and 2) was fitted to the data applying the drc modlFit function:

$$Y = c + \frac{d-c}{1+\exp[b(\log(x)-\log(ED_{50}))]} \quad (3.1)$$

$$Y = \frac{d}{1+\exp[b(\log(x)-\log(ED_{50}))]} \quad (3.2)$$

Y represents the response variable (shoot dry weight, plant height, coleoptile, and hypocotyl lengths), *d* represents the coefficient corresponding to the upper limit, *x* represents herbicide dose, ED_{50} is the dosage cause 50% reduction and *b* determine the shape of the curve.

Figure 3.14 illustrates the resistance indices (*RI's*), the ratio between the estimated ED_{50} value of R and S populations. To differentiate between herbicide doses, the result for each experiment was analyzed separately using ANOVA in Minitap16 software and the means were compared using the Tukey test at a significant level = 0.05.

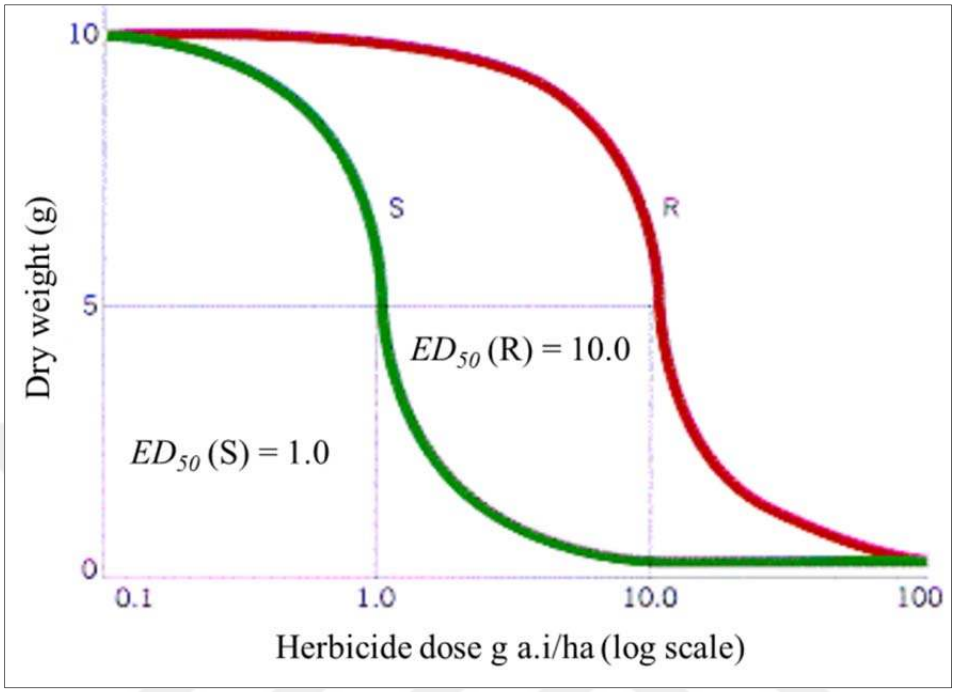

Figure 3.14. Dose-response curves for susceptible (S) and resistant (R) populations of sterile wild oat (*Avena sterilis* L.).

$$\text{Resistance index (RI)} = \frac{ED_{50}\ (R)}{ED_{50}\ (S)} = \frac{10.0}{1.0} = 10$$

4. RESULTS AND DISCUSSION

4.1. Quick-Test for Detecting Herbicide Resistance in *Avena sterilis* L.

4.1.1. Agar-Based Seed Germination Test

Plant agar at 0.8% was proved to be good for the germination of *Avena sterilis* in the laboratory conditions, and there is no need to add any chemicals to avoid contamination with fungus. Seeds were considered to have germinated when radicle or hypocotyl was approximately 2mm or more. Seeds were considered not germinated or dead when radicle or hypocotyl protruded less than 2 mm (Hull and Moss, 2007). Based on this, the average percentage seed germination of resistance (R) and susceptible (S) populations of *A. sterilis* was higher than 95% in untreated control without any additional treatment to enhance the germination, and no difference in seed germination between the first and second experiment was found. In all herbicide treatments, the germination started within 5 days of incubation at 10^{o}C, and the maximum germination (90%) occurred in untreated control 7 days after incubation. By increasing the herbicide concentrations, the seed germination was decreased in R and S populations. More than 20% germination in both populations was found at the higher concentration of clodinafop-propargyl, mesosulfuron-methyl, and pyroxsulam, whereas less than 10% was germinated in R and S populations treated with pinoxaden (Figure 4.1). The most informative dosage (the dosage that provided the greatest difference in germination between R and S) was 9.6, 1.8, and 1.2 µM for clodinafop-propargyl, pinoxaden and mesosulfuron-methyl, respectively, no difference was found between R and S in all concentrations of pyroxsulam. Hull and Moss (2007) provided that inhibition in seed germination of black-grass (*Alopecurus myosuroides*) at the higher dose of mesosulfuron-methyl will not prevent germination of S biotype. However, germination will be considerably less compared to R population.

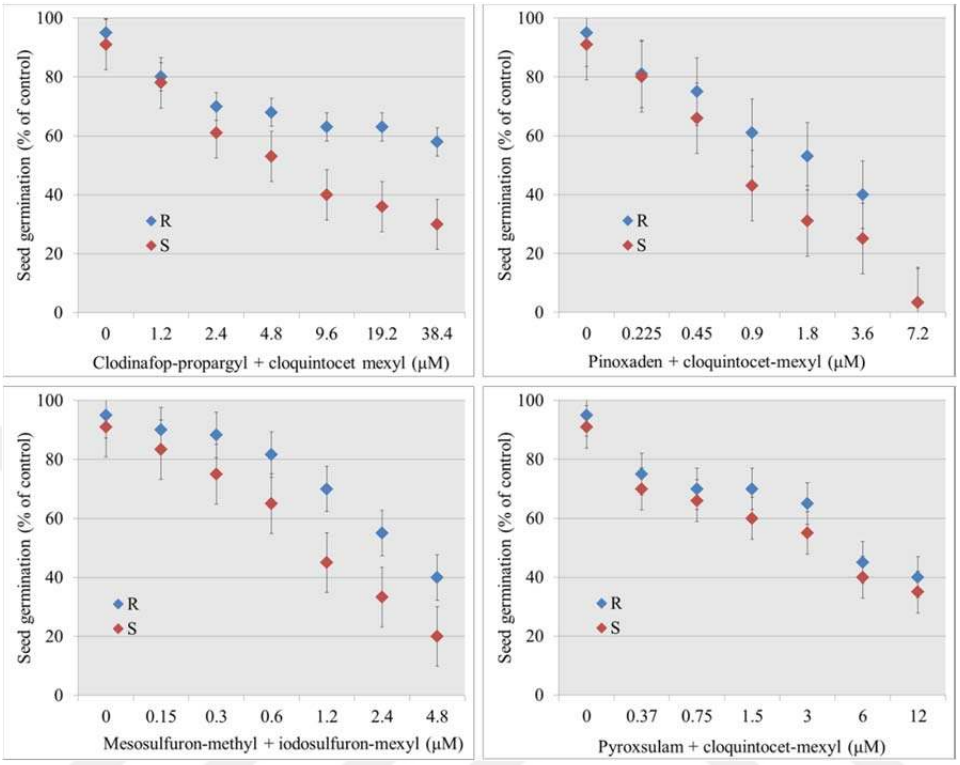

Figure 4.1. Effect of increasing herbicide concentrations on germination of resistance (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) Vertical bars are a standard error (±SE).

Herbicide effect on radicle and hypocotyl lengths was obvious when they started germination and after 10 days of incubation, the difference was even more pronounced between the R and S populations. Clodinafop-propargyl and pinoxaden caused inhibition on radicle elongation, and an increased concentration, the hypocotyl growth was stunted in R and S populations than what did in the untreated control. Figure 4.2 and Figure 4.3 showed the effect of different concentrations of clodinafop-propargyl and pinoxaden on radicle and hypocotyl length of R and S populations.

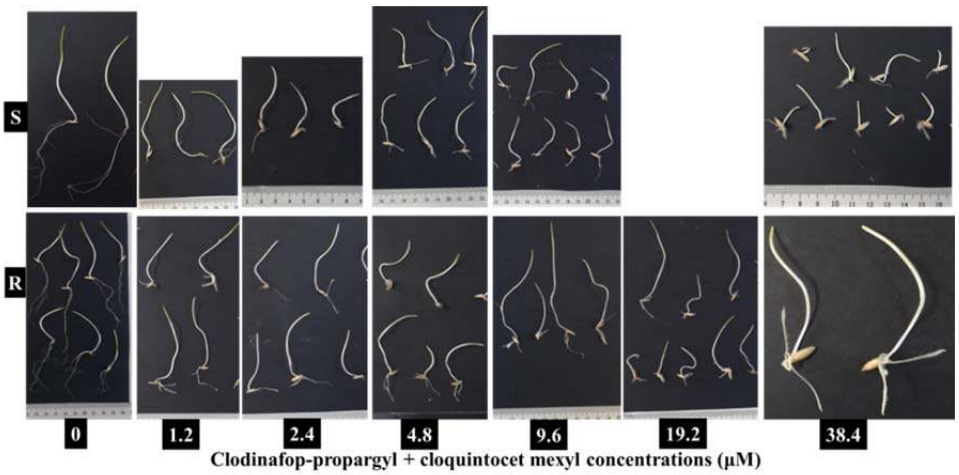

Figure 4.2. Effect of clodinafop-propargyl on hypocotyl-and radicle-length of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.)

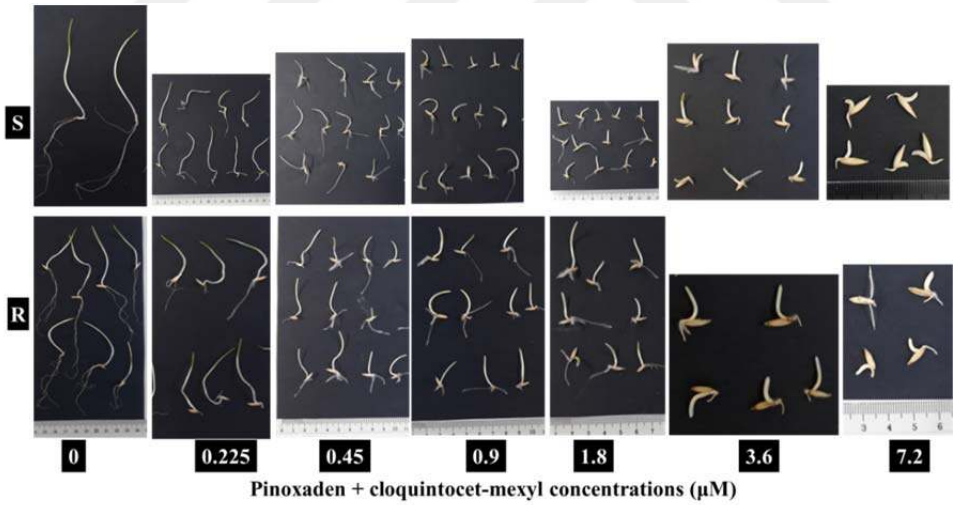

Figure 4.3. Effect of pinoxaden on hypocotyl-and radicle-length of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.)

The effect of pinoxaden on radicle length was far more severe than on hypocotyl length. Even at low concentration, a very short radicle was observed for the seedlings of the S population. It is obvious that ACCase-inhibiting herbicides were more effective on radicle length than on the hypocotyl elongation in *A. sterilis*. A similar reduction in radicle length was observed on *Phalaris minor* treated with fenoxaprop-P (Tal *et al.*, 2000), and extreme reduction in radicle length of the seedlings of *Sinapis arvensis* and *Brassica napus* subjected to different rates of auxinic herbicides such as dicamba and 2,4-D was observed by Wei *et al.* (2000) and Polit *et al.* (2014). However, these differences may indicate the existence of the resistance gene, in different parts of tissue and their sensitivity toward herbicides was different (Tal *et al.*, 2000). From these observations, the study suggests that the use of ACCase herbicide in the field could be minor as post-emergence.

The response of R and S populations to mesosulfuron-methyl and pyroxsulam (ALS) was different from the response to ACCase herbicides. ALS-inhibiting herbicide affected the hypocotyl elongation than the radicle (Figure 4.4 and 4.5). The length of radicle and hypocotyl for the R population was almost unaffected by the high concentration of mesosulfuron-methyl compared to S population. The length of radicle and hypocotyl in both R and S populations treated with ALS herbicide was significantly greater than those populations treated with ACCase herbicides. Similar observations have been seen in rigid ryegrass (*Lolium rigidum*) treated with sulfometuron (Burnet *et al.*, 1994). However, the hypocotyl of the S population was particularly sensitive to pyroxsulam compared with the radicle, confirming that the R population was really resistant to herbicides than the S population. In this test, radicle or hypocotyl length is the appropriate parameter to assess ALS herbicide resistance (Xu *et al.*, 2010), and hypocotyl length was appropriate to evaluate the response to ACCase herbicide (Burke *et al.*, 2006).

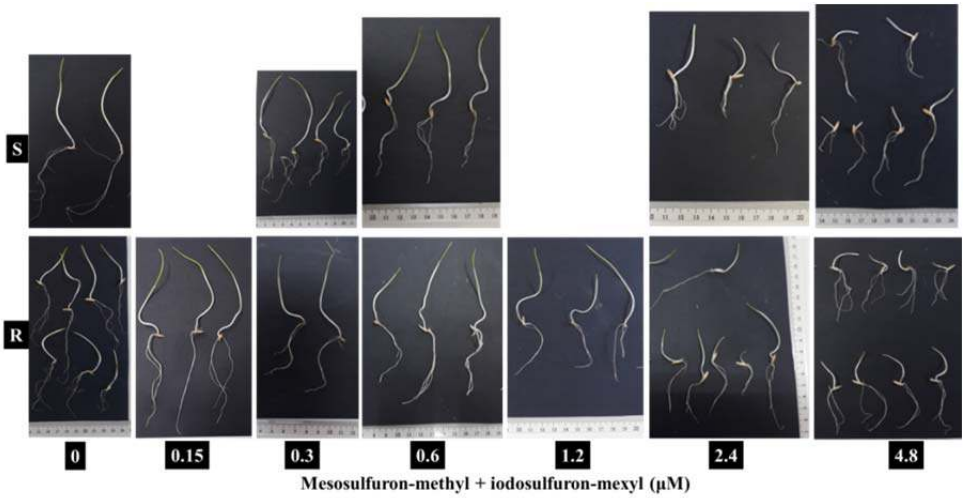

Figure 4.4. Effect of mesosulfuron-methyl on hypocotyl-and radicle-length of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.)

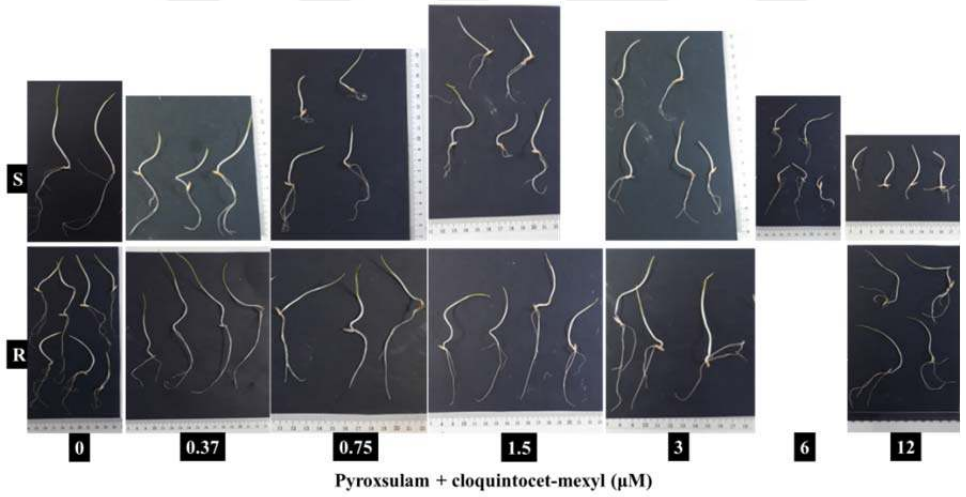

Figure 4.5. Effect of pyroxsulam on hypocotyl-and radicle-length of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.)

From these differences between the radicle and hypocotyl length, we concluded that the activity of ACCase and ALS herbicide was not affected by agar medium in glass Petri-dishes and light, which may explain the increased variability

observed. Beckie *et al.* (1990) found a yellowish tinge on plastic dishes after trifluralin used, and it was considered the chemical properties of some herbicide may be affected by plastic-dishes.

The non-linear model provided a good explanation of the association between the length of the radicle and hypocotyl of *A. sterilis* and clodinafop-propargyl rates. The ED_{50} values of R and S populations in the first and second experiment were quite different and resistance indices of ≥ 2 and ≥ 3 were found in radicle and hypocotyl, parameters, respectively (Table 4.1).

Table 4.1. Regression parameters estimated from the agar-based seed test based on hypocotyl and radicle length (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl+cloquintocet-mexyl

			Regression parameters					
	Exp no	Pop	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)	*p-values*	R^2	*RI*
Hypocotyl	1	S	100.1 (5.7)	0.73 (0.1)	1.58 (0.4)	***	0.99	
		R	100.3 (5.7)	0.54 (0.1)	4.78 (1.4)	**	0.99	3.00
	2	S	99.8 (5.0)	0.98 (0.1)	4.89 (0.8)	***	0.99	
		R	99.3 (4.9)	0.91 (0.1)	11.71 (2.1)	***	0.99	2.39
Radicle	1	S	99.1 (6.7)	1.06 (0.1)	2.49 (0.5)	***	0.98	
		R	98.9 (6.6)	1.03 (0.1)	4.97 (1.0)	***	0.99	1.99
	2	S	101.0 (8.7)	0.63 (0.1)	8.10 (3.2)	*	0.99	
		R	101.3 (8.6)	0.57 (0.1)	12.08 (5.3)	*	0.99	1.49

*Significant codes: *(p ≤ 0.05), **(p ≤ 0.01), ***(p=0.001); R^2=coefficient of determination

For each resistant and susceptible population, there was a general reduction in radicle and hypocotyl length as clodinafop-propargyl concentration increased relative to untreated controls (Figure. 4.6).

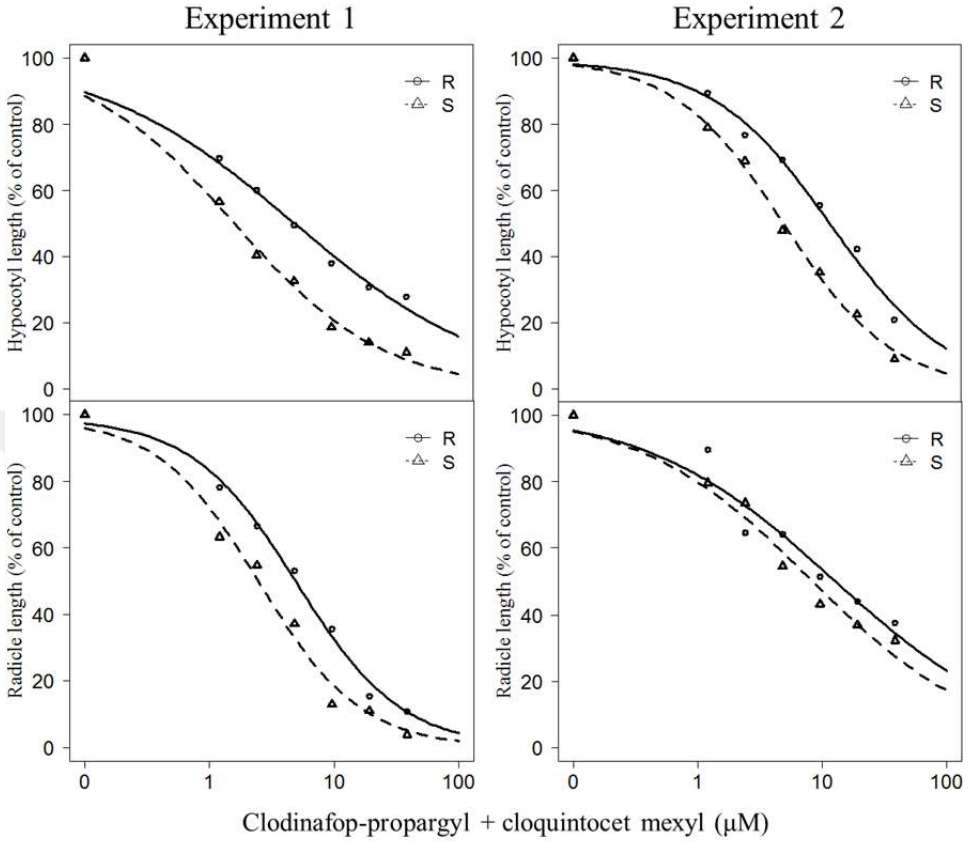

Figure 4.6. Dose-response curves of radicle and hypocotyl response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl+cloquintocet-mexyl.

The calculated ED_{50} values from radicle and hypocotyl length treated by pinoxaden in the R and S populations were shown in Table 4.2. The resistance index values showed that the radicle was 5-fold more resistant compared to the S population at the second experiment. The estimated RI on hypocotyl was very close in both experiments.

Table 4.2. Regression parameters estimated from the agar-based seed test based on hypocotyl and radicle length (%) of resistant (R) and susceptible (S)

populations of sterile wild oat (*Avena sterilis* L.) treated with pinoxaden+cloquintocet-mexyl

	Exp no	Pop	Regression parameters			p-values	R^2	RI
			d (±SE)	*b* (±SE)	ED_{50} (±SE)			
Hypocotyl	1	S	99.6 (8.0)	0.81 (0.1)	0.39 (0.1)	**	0.98	
		R	97.1 (8.2)	0.99 (0.2)	1.17 (0.3)	***	0.99	3.00
	2	S	101.4 (6.0)	1.06 (0.1)	0.64 (0.1)	***	0.99	
		R	97.5 (6.3)	0.92 (0.1)	1.92 (0.4)	***	0.98	2.97
Radicle	1	S	99.1 (6.2)	1.2 (0.2)	0.67 (0.1)	***	0.99	
		R	93.8 (5.3)	1.6 (0.3)	1.86 (0.2)	***	0.97	2.76
	2	S	101.6 (5.5)	0.84 (0.1)	0.61 (0.1)	***	0.95	
		R	97.7 (5.7)	0.81 (0.1)	3.21 (0.7)	***	0.98	5.24

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

Log-logistic analysis of radicle and hypocotyl length data sufficiently described the response of R and S lines to pinoxaden with R^2 0.95–0.99. The largest difference in radicle elongation between the R and S populations found at pinoxaden concentrations of 1 to 10 µM (Figure 4.7) and the radicle length of the S population was not completely inhibited. The largest difference in hypocotyl length between the R and S populations was measured at 0.1 to 1 µM, this difference was less than that detected for radicle length.

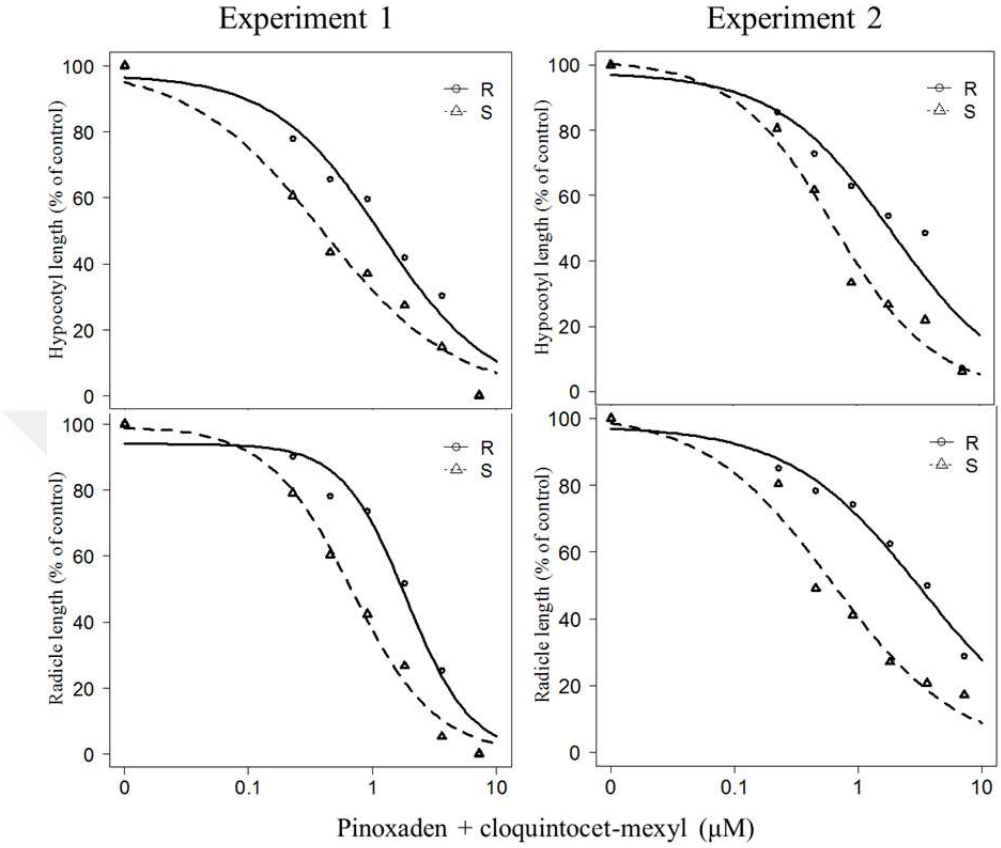

Figure 4.7. Dose-response curves of radicle and hypocotyl response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with pinoxaden+cloquintocet-mexyl.

The estimated ED_{50} of mesosulfuron-methyl in radicle was 2.67-fold and in hypocotyl was 2.1-fold. There was no difference in the susceptibility of S populations in the first and the second experiment (Table 4.3).

Table 4.3. Regression parameters estimated from the agar-based seed test based on hypocotyl and radicle length (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with mesosulfuron-methyl+iodosulfuron

			Regression parameters					
	Exp no	Pop	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)	*p-values*	R^2	*RI*
Hypocotyl	1	S	98.7 (8.8)	0.60 (0.1)	0.76 (0.3)	*	0.99	
		R	98.2 (8.8)	0.70 (0.1)	1.21 (0.6)	*	0.99	1.58
	2	S	99.5 (5.6)	0.95 (0.1)	0.65 (0.1)	***	0.99	
		R	98.2 (5.7)	0.82 (0.1)	1.37 (0.3)	***	0.99	2.10
Radicle	1	S	102.0 (8.9)	0.89 (0.2)	0.72 (0.2)	**	0.99	
		R	101.4 (8.5)	0.89 (0.2)	1.95 (.6)	**	0.99	2.67
	2	S	102.5 (5.0)	0.78 (0.1)	0.78 (0.1)	***	0.99	
		R	100.7 (4.9)	0.85 (0.1)	1.80 (0.3)	***	0.99	2.29

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

Both radicle and hypocotyl lengths of the S population were inhibited at 0.65 to 0.78 µM of mesosulfuron-methyl, whereas those of the R population were only slightly decreased at the highest dose of mesosulfuron-methyl (Figure 4.8).

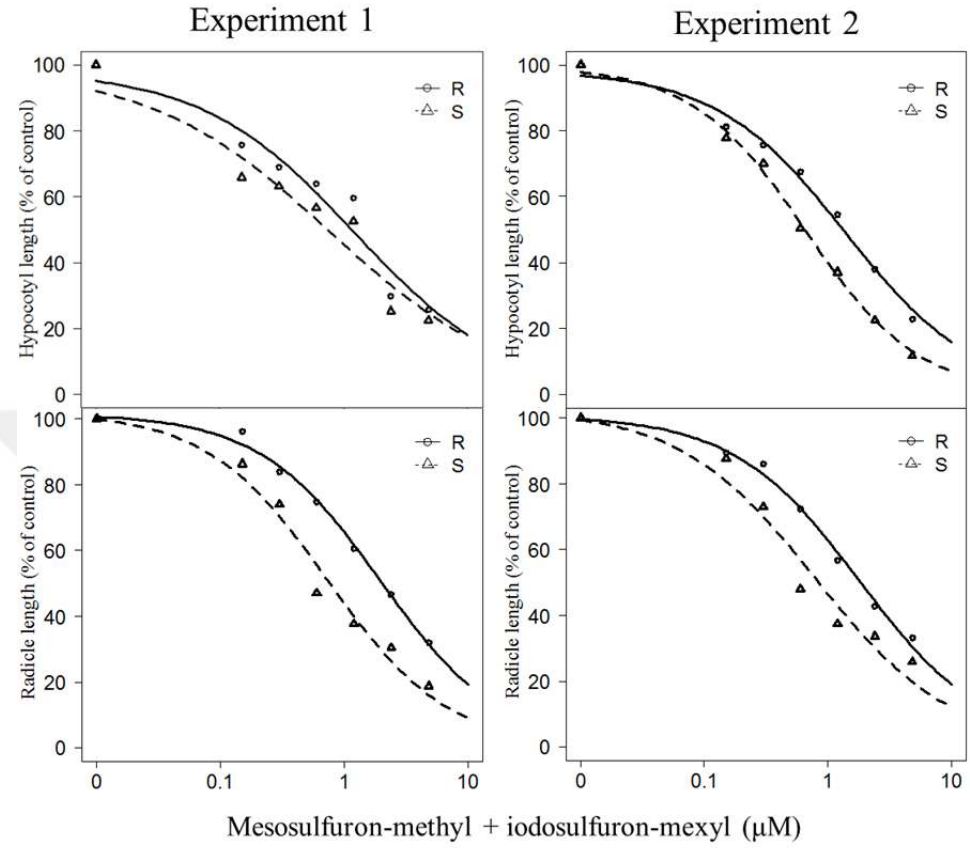

Figure 4.8. Dose-response curves of radicle and hypocotyl response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with mesosulfuron-methyl+iodosulfuron.

The ED_{50} values of pyroxsulam in R and S populations in the first and second experiment are given in Table 4.4. The resistance index was 3.32-fold and 1.58-fold for radicle and hypocotyl, respectively. Severe reduction in hypocotyl length in the S population occurred in the first experiment (Figure 4.9).

Table 4.4. Regression parameters estimated from the agar-based seed test based on hypocotyl and radicle length (%) of resistant (R) and susceptible (S)

populations of sterile wild oat (*Avena sterilis* L.) treated with pyroxsulam+cloquintocet-mexyl

	Exp no	Pop	Regression parameters					
			d (±SE)	*b* (±SE)	ED_{50} (±SE)	*p-values*	R^2	*RI*
Hypocotyl	1	S	99.5 (7.1)	0.94 (0.1)	0.75 (0.1)	***	0.99	
		R	99.7 (7.0)	0.66 (0.1)	1.19 (0.7)	**	0.99	1.58
	2	S	99.9 (8.3)	0.66 (0.1)	1.99 (0.7)	**	0.99	
		R	99.1 (8.4)	0.68 (0.1)	3.05 (1.1)	**	0.99	1.53
Radicle	1	S	100.2 (7.9)	0.60 (0.1)	1.28 (0.4)	**	0.99	
		R	100.7 (7.7)	0.62 (0.1)	3.28 (1.2)	**	0.98	2.55
	2	S	101.7 (5.9)	0.65 (0.1)	2.29 (0.6)	***	0.99	
		R	101.4 (5.7)	0.68 (0.1)	7.62 (2.1)	***	0.99	3.32

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

The radicle and hypocotyl elongation of the R population, when exposed to ALS, was not inhibited, whereas complete inhibition of radicle was observed for the S population treated with ACCase herbicide. These results indicate that the agar-based seed germination test can accurately and reliably detect herbicide resistance in *A. sterilis* population. Using this test can also help to collect samples rapidly and informed of any potential problem. Furthermore, the method could be utilized for the determination of herbicide resistance in a short time and enable farmers to make decisions to start effective weed management practices to control of the ACCase and ALS-inhibiting herbicide-resistant *A. sterilis*.

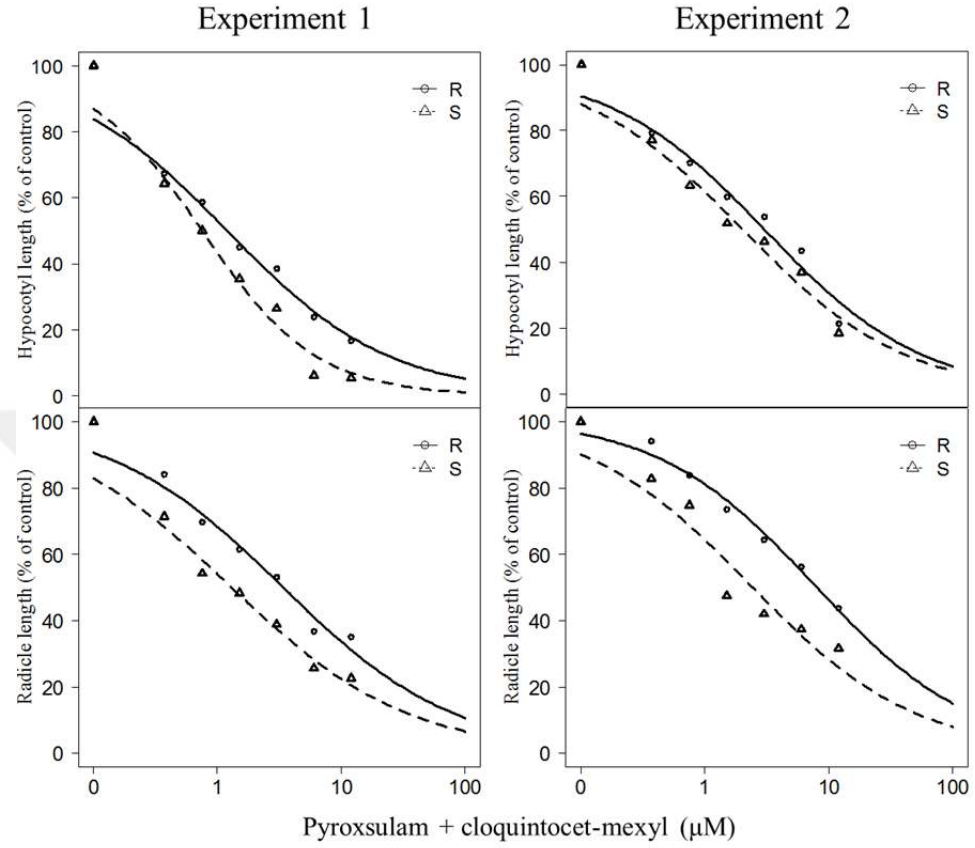

Figure 4.9. Dose-response curves of radicle and hypocotyl response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with pyroxsulam+cloquintocet-mexyl.

4.1.2. Agar-Based Seedling Test

Plant agar (0.8% w/v) proved a suitable substrate for *A. sterilis* for growing seedlings under the same greenhouse conditions as a classical pot test (Kaundun *et al.*, 2011b). Table 4.5 summarized the parameters obtained from the seedling assay. Based on visual assessments, resistance was observed in the R population which was 2.39 times more resistant than the S population to clodinafop-propargyl relative to the untreated control. At high concentration of clodinafop-propargyl S population was completely dead while 20% of the R population was survived. For

mesosulfuron-methyl + iodosulfuron, both R and S population were susceptible, showing the same ED_{50} values (0.31) and at high concentration of mesosulfuron-methyl+iodosulfuron 15% of R and S populations were still surviving (Figure 4.10). Some plants remained injured or began to senesce without any indication of new growth (Figure 4.11 and 4.12).

Table 4.5. Regression parameters estimated from the agar-based seedling test on visually assessed survival (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl+cloquintocet-mexyl and mesosulfuron-methyl+iodosulfuron

		Regression parameters					
Herbicides	Pop	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)	*p-values*	R^2	*RI*
clodinafop-propargyl	S	99.7 (12.5)	1.19 (0.3)	2.65 (0.9)	**	0.99	
	R	100.0 (0.0)	0.96 (0.3)	6.35 (2.6)	*	0.99	2.39
mesosulfuron-methyl + iodo	S	100.0 (0.0)	1.06 (0.2)	0.31 (0.06)	***	0.99	
	R	99.9 (1.0)	0.98 (0.2)	0.31 (0.07)	***	0.99	1.00

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

However, the transplanted seedlings at 2-3 leaf stage were very sensitive to mesosulfuron-methyl + iodosulfuron-methyl. In contrast to previous studies, herbicide treatments at seedling stage under greenhouse conditions can have a profound impact on the level of resistance in the targeted weed species (Dinelli *et al.*, 2006; Shrestha *et al.*, 2007).

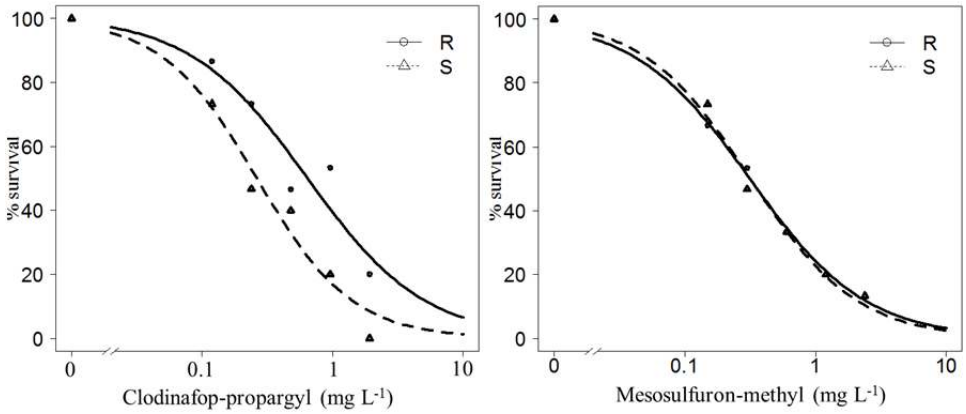

Figure 4.10. Dose-response curves for resistance (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl and mesosulfuron-methyl+iodosulfuron-methyl in the seedling assay.

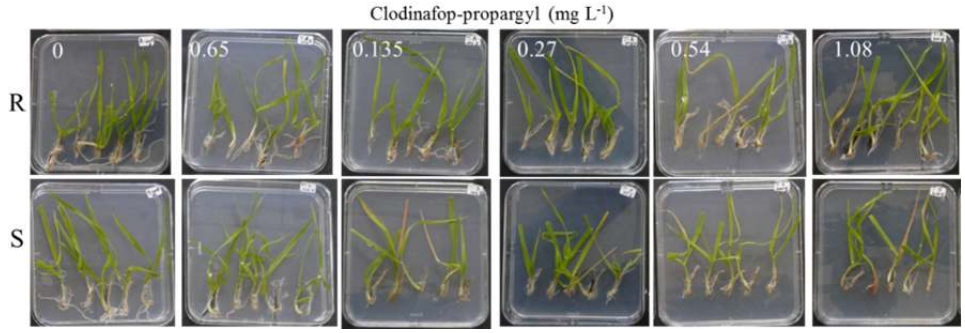

Figure 4.11. Typical resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) at the different rates of clodinafop-propargyl in the agar-based seedling test.

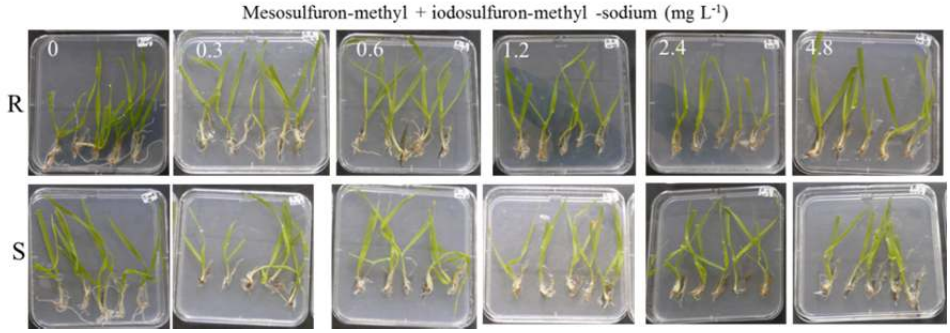

Figure 4.12. Typical resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) at the different rates of mesosulfuron-methyl+iodosulfuron-methyl-sodium in the agar-based seedling test.

4.1.3. Filter Paper-Based Seed Germination Test

ACCase-inhibiting herbicides symptoms appeared on both S and R populations at 14 and 21 days after treatment, respectively. At the high dose of clodinafop-propargyl (384g a.i/ha), 70% and 100% symptoms were observed in the R and S populations, respectively, on the 28th day after treatment (DAT). A high rate of pinoxaden (324g a.i/ha) 100% symptom in S and R populations were observed on the 21 and 28 DAT, respectively, (Figure 4.13). From the observations, the efficacy of clodinafop-propargyl was started earlier than that resulted from the pinoxaden application. In general, a lower dose of both herbicides caused interveinal bleaching (whitening) and at high doses, the plant was turned yellow.

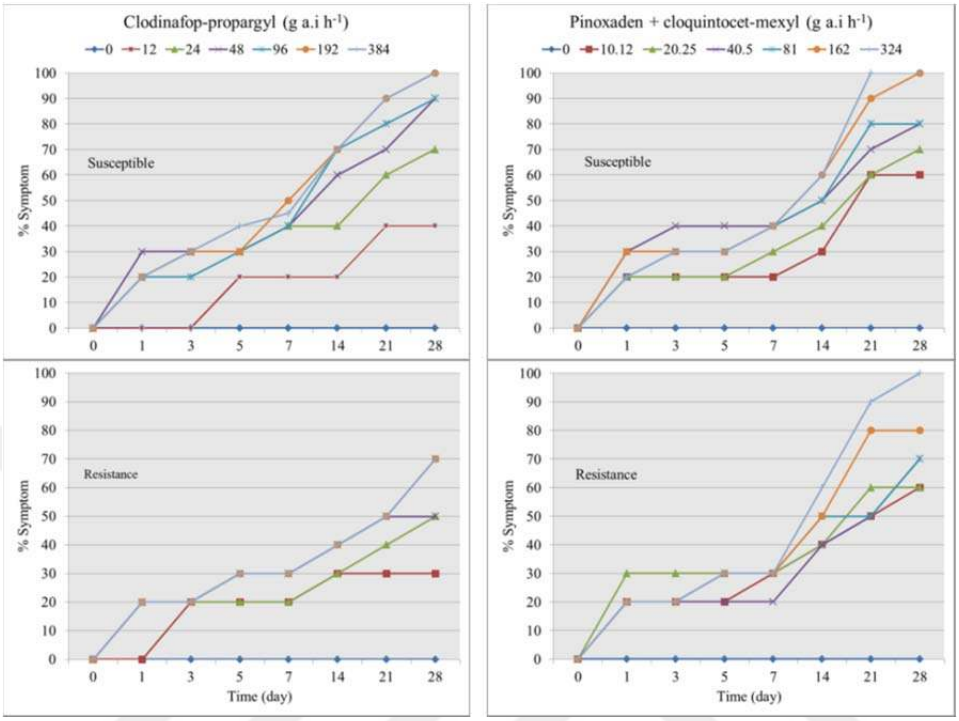

Figure 4.13. Percentage symptoms of clodinafop-propargyl and pinoxaden in resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) on several days of treatment.

For mesosulfuron-methyl+iodosulfuron (9g a.i/ha), 50% symptom in S and 30% in R were observed on the 14 and 28 DAT, respectively. At high dose, all S plants were killed on the 21 DAT, but 50% of R plants were surviving at 28 DAT. Fore pyroxsulam + cloquintocet-mexyl (18.75g a.i/ha), 50% symptoms in S and 40% of the R population were observed on the 14 and 21 DAT, respectively. At high dose pyroxsulam + cloquintocet-mexyl (150g a.i/ha) S plants were completely killed at 21 DAT, and 80% symptoms were occurring in R plants (Figure 4.14). In general, ALS-inhibiting herbicides caused injury for several DAT. The symptoms began with chlorosis in leaves and became necrotic, stunting, and distortion within several days of treatment.

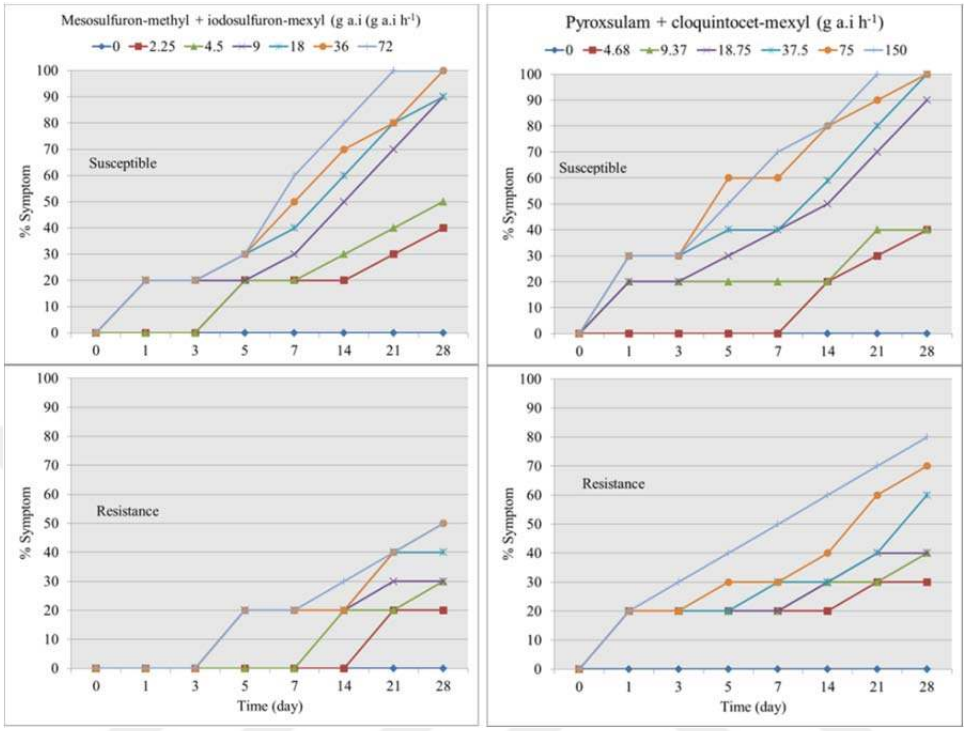

Figure 4.14. Percentage symptoms of mesosulfuron-methyl+iodosulfuron and pyroxsulam + cloquintocet-mexyl in resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) in several days of treatment.

Full dose-response curves for R and S populations were obtained based on plant height. For clodinafop-propargyl, in four experiments ANOVA test showed a significant difference between S and R populations (p-value=0.001). The four-parametric logistic model fitted the data well ($R^2 \leq 0.99$) in the first and the second experiment. Three parametric models were fitted in third and fourth experiments on the 28 DAT. The highest ED_{50} values of clodinafop-propargyl were 86.51 and 252.03g a.i ha^{-1} for the S and R populations, respectively, indicating that the R population was 2.91 times more resistant than S populations (Table 4.6, Figure 4.15). Differences in ED_{50} in four experiments indicated that the

susceptibility of individual plant within the population or herbicide activity may be affected by the experimental conditions.

Table 4.6. Regression parameters estimated from the filter paper-based test based on plant height (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl+cloquintocet-mexyl

Exp no	Pop	Regression parameters				p-val	R^2	RI
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)			
1	S	20.5 (3.7)	99.9 (2.3)	1.00 (0.1)	17.46 (2.3)	***	0.99	
	R	17.1 (23.4)	100.0 (2.3)	0.55 (0.1)	78.20 (86.5)		0.99	4.47
2	S	24.4 (2.8)	99.5 (2.5)	1.68 (0.2)	32.13 (2.8)	***	0.99	
	R	23.5 (10.5)	100.5 (2.5)	0.85 (0.1)	60.39 (24.0)	*	0.97	1.87
3	S	-	102.6 (2.8)	0.83 (0.0)	86.51 (9.0)	***	0.99	
	R	-	102.6 (2.6)	0.86 (0.0)	252.03 (27.4)	***	0.98	2.91
4	S	-	104.1 (2.9)	0.84 (0.0)	64.90 (6.9)	***	0.98	
	R	-	103.5 (2.8)	0.81 (0.0)	150.64 (17.3)	***	0.99	2.32

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

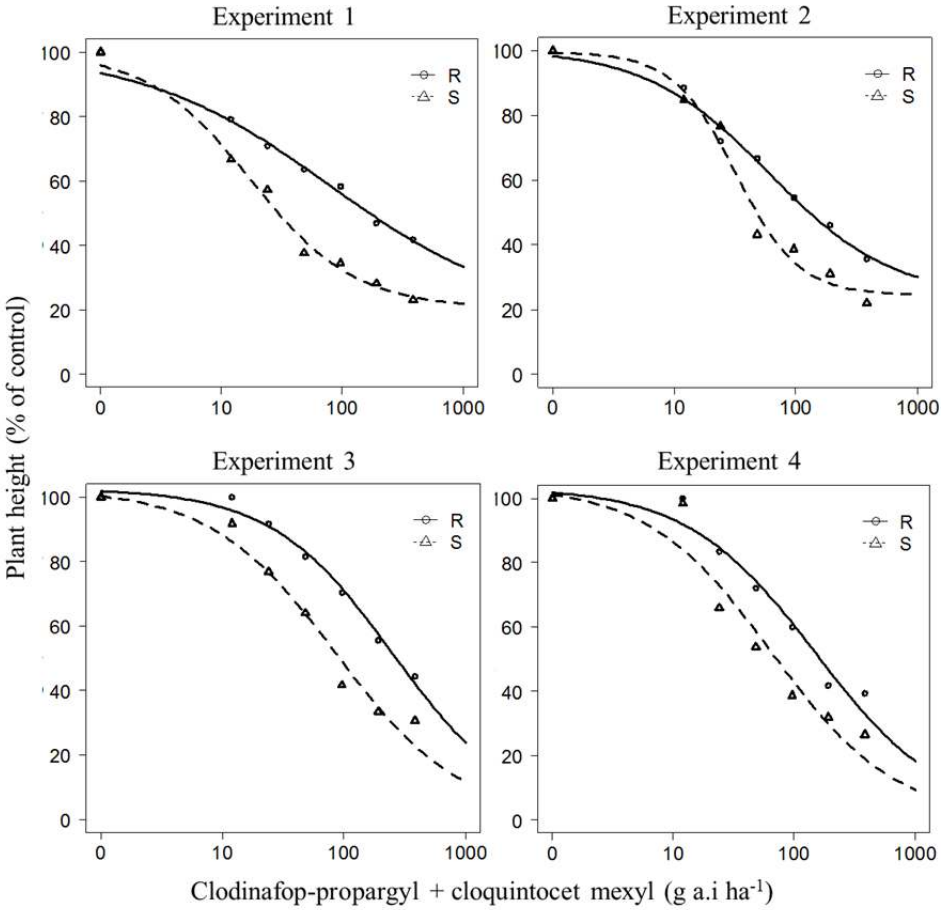

Figure 4.15. Plant height response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of clodinafop-propargyl+cloquintocet-mexyl.

In the population treated with Pinoxaden+cloquintocet-mexyl, in four experiments ANOVA test showed a significant difference between S and R populations (p-value=0.001, $R^2 \leq 0.99$). The highest estimated ED_{50} values were 63.30 and 156g a.i/ha in S and R populations, respectively, that were founded on experiment three with resistance index (RI) 2.46-fold. The lowest estimated ED_{50} values were 25.57 g a.i/ha in S and 62.11 g a.i/ha for R population, resulted in RI of 2.42-fold occurred in the second experiment (Table 4.7, Figure 4.16).

Table 4.7. Regression parameters estimated from the filter paper-based test based on plant height (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with Pinoxaden+cloquintocet-mexyl

Exp no	Pop	Regression parameters				p-val	R^2	RI
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)			
1	S	-	100.0 (0.0)	0.68 (0.0)	30.02 (3.2)	***	0.99	
	R	-	99.8 (2.5)	0.57 (0.0)	119.17 (15.6)	***	0.99	3.96
2	S	8.8 (2.7)	100.0 (2.4)	1.38 (0.1)	25.57 (2.1)	***	0.99	
	R	25.7 (7.6)	99.9 (2.3)	1.10 (0.1)	62.11 (14.8)	***	0.97	2.42
3	S	-	100.8 (2.4)	0.69 (0.0)	63.30 (6.7)	***	0.99	
	R	-	98.3 (2.3)	0.94 (0.0)	156.17 (13.4)	***	0.98	2.46
4	S	-	100.0 (0.0)	1.27 (0.0)	48.82 (3.3)	***	0.98	
	R	-	99.7 (1.8)	1.71 (0.1)	106.02 (5.5)	***	0.99	2.17

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

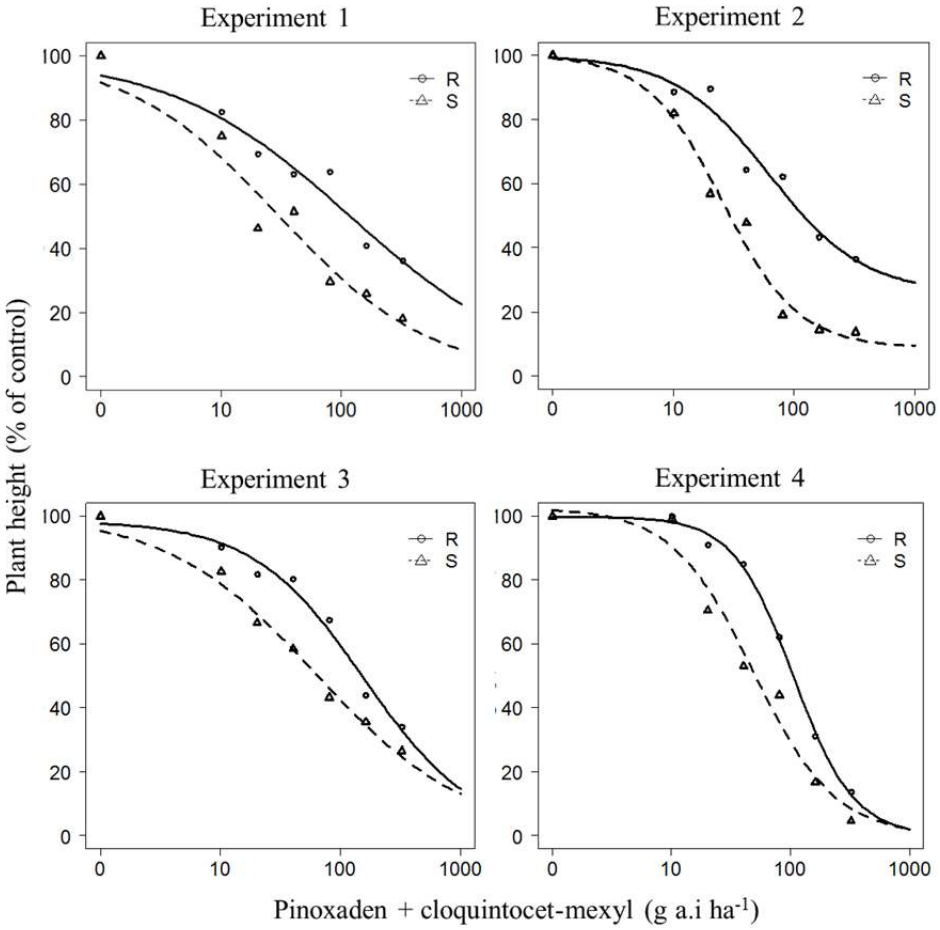

Figure 4.16. Plant height response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of Pinoxaden+cloquintocet-mexyl.

In the tested population with mesosulfuron-methyl+iodosulfuron, the highest estimated ED_{50} values were 14.40 and 58.00g a.i/ha for S and R populations, respectively, resulted in RI of 4.02-fold, which was founded on experiment 1. The lowest ED_{50} values of S and R populations were found on experiment two with RI of 3.05 (Table 4.8, Figure 4.17).

Table 4.8. Regression parameters estimated from the filter paper-based test based on plant height (%) of resistant (R) and susceptible (S) populations of

sterile wild oat (*Avena sterilis* L.) treated with mesosulfuron-methyl+iodosulfuron

Exp no	Pop	Regression parameters				p-val	R^2	RI
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)			
1	S	-	100.0 (0.0)	1.01 (0.0)	14.40 (1.1)	***	0.99	
	R	-	99.8 (2.3)	0.87 (0.0)	58.00 (5.7)	***	0.99	4.02
2	S	41.0 (1.0)	100.0 (0.0)	1.72 (0.4)	1.40 (0.2)	***	0.99	
	R	51.6 (2.2)	100.0 (0.0)	1.19 (0.1)	4.30 (0.5)	***	0.97	3.05
3	S	19.5 (2.9)	100.0 (0.0)	1.25 (0.1)	5.19 (0.5)	***	0.99	
	R	17.7 (7.6)	100.0 (0.0)	1.03 (0.1)	14.34 (3.2)	***	0.98	2.75
4	S	28.8 (8.7)	99.9 (2.8)	0.69 (0.2)	3.25 (1.2)	**	0.98	
	R	47.1 (6.3)	100.0 (0.0)	0.84 (0.2)	3.89 (1.3)	**	0.99	1.19

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

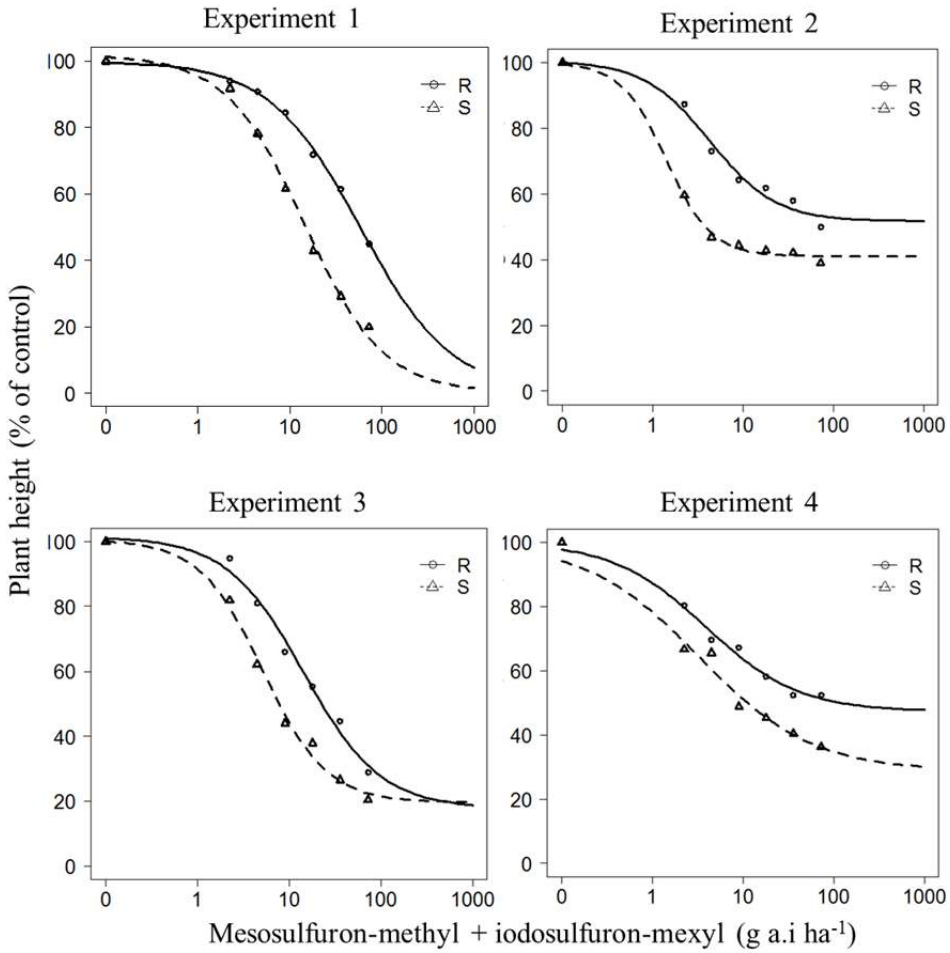

Figure 4.17. Plant height response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of mesosulfuron-methyl+iodosulfuron.

For pyroxsulam+cloquintocet-mexyl, the highest estimated ED_{50} values were 45.51 and 79.40g a.i/ha for S and R populations, respectively, resulted in RI of 1.74-fold, which was founded in experiment 3. The lowest estimated ED_{50} values of S and R populations were found in experiment 1 with RI of 4.20-fold (Table 4.9, Figure 4.18).

Table 4.9. Regression parameter estimated from the filter paper-based test based on plant height (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with pyroxsulam+cloquintocet-mexyl

Exp no	Pop	Regression parameters				P-val	R^2	RI
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)			
1	S	29.9 (4.1)	100.5 (2.6)	1.14 (0.2)	8.16 (1.2)	***	0.99	
	R	25.2 (13.7)	100.6 (2.5)	0.87 (0.2)	34.29 (17.0)	*	0.99	4.20
2	S	-	102.8 (3.5)	1.72 (0.4)	17.01 (0.1)	***	0.99	
	R	-	102.7 (3.0)	1.19 (0.1)	85.99 (0.1)	***	0.97	5.05
3	S	-	102.8 (3.7)	0.65 (0.0)	45.51 (7.8)	***	0.99	
	R	-	101.8 (3.3)	0.94 (0.1)	79.40 (10.0)	***	0.98	1.74
4	S	-	102.8 (4.2)	0.60 (0.0)	24.38 (4.7)	***	0.98	
	R	-	101.2 (4.2)	0.71 (0.0)	51.41 (9.3)	***	0.99	2.10

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

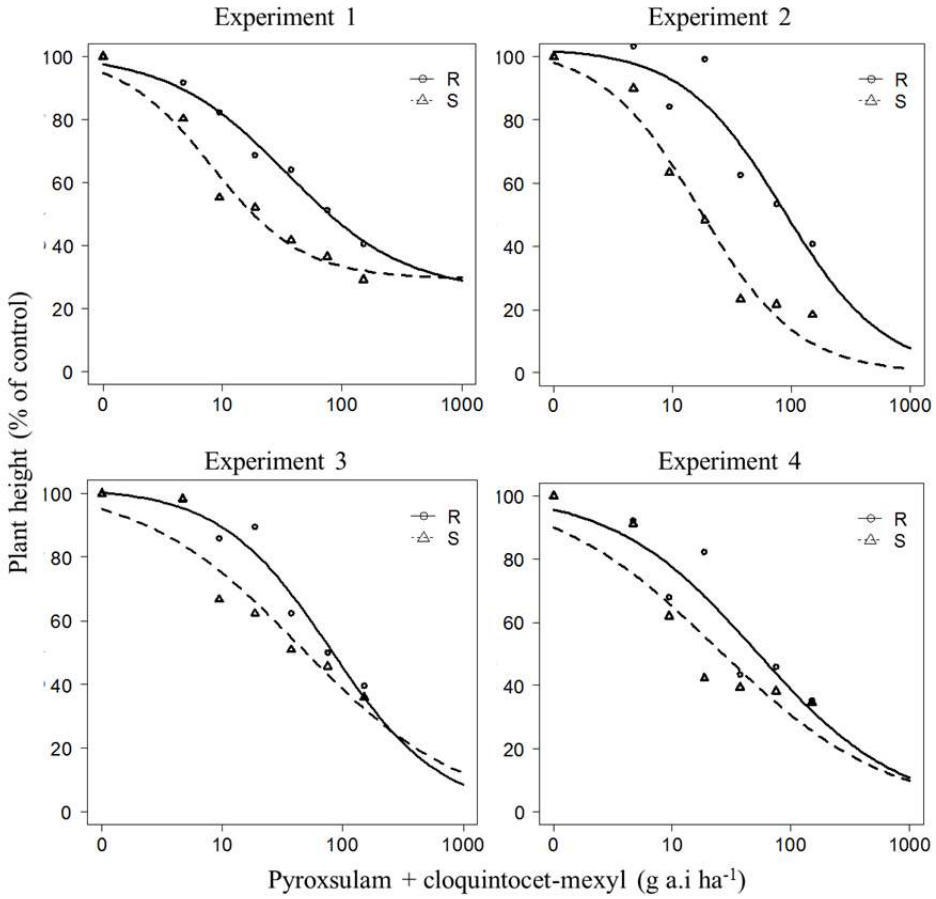

Figure 4.18. Plant height response of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of pyroxsulam+cloquintocet-mexyl.

4.2. Greenhouse Test for Detecting Herbicide Resistance in *Avena sterilis* L.

In the population treated with clodinafop-propargyl, calculated ED_{50} values for S and R populations in each of the four experiments were illustrated in Table 4.10. The lowest ED_{50} values of S population was 1.80g a.i ha^{-1} and for R population was 21.56g a.i ha^{-1} resulted in RI of 11.93 times more resistant than the S population, founded on experiment 2. All plants in R and S populations were not killed at the high dose of clodinafop-propargyl in the four experiments. It is to be

noted that only 10% of S and above 20% of R plants survived at 384 g a.i ha^{-1} of the four experiments (Figure 4.19 and 4.20).

Table 4.10. Regression parameters estimated from the greenhouse test based on dry weight (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl+cloquintocet-mexyl

Exp no	Pop	Regression parameters					
		d (±SE)	*b* (±SE)	ED_{50} (±SE)	*p-values*	R^2	*RI*
1	S	99.5 (9.3)	0.99 (0.3)	13.79 (4.9)	**	0.99	
	R	99.5 (9.3)	0.56 (0.1)	52.79 (24.9)	*	0.99	3.82
2	S	83.0 (9.2)	0.35 (0.1)	1.80 (3.4)		0.99	
	R	69.6 (9.3)	0.53 (0.1)	21.56 (11.9)		0.97	11.93
3	S	100.2 (9.4)	1.03 (0.2)	51.80 (15.5)	**	0.99	
	R	97.6 (8.8)	0.78 (0.2)	225.86 (91.1)	*	0.98	4.35
4	S	99.7 (8.4)	0.72 (0.1)	61.26 (21.5)	**	0.98	
	R	90.24 (6.2)	2.31 (1.1)	138.64 (22.6)	***	0.99	2.26

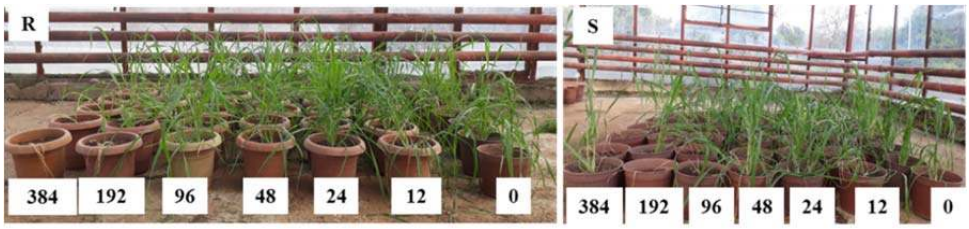

Figure 4.19. Effect of clodinafop-propargyl+cloquintocet-mexyl (g a.i ha^{-1}) on resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.), on the 28th day after treatment.

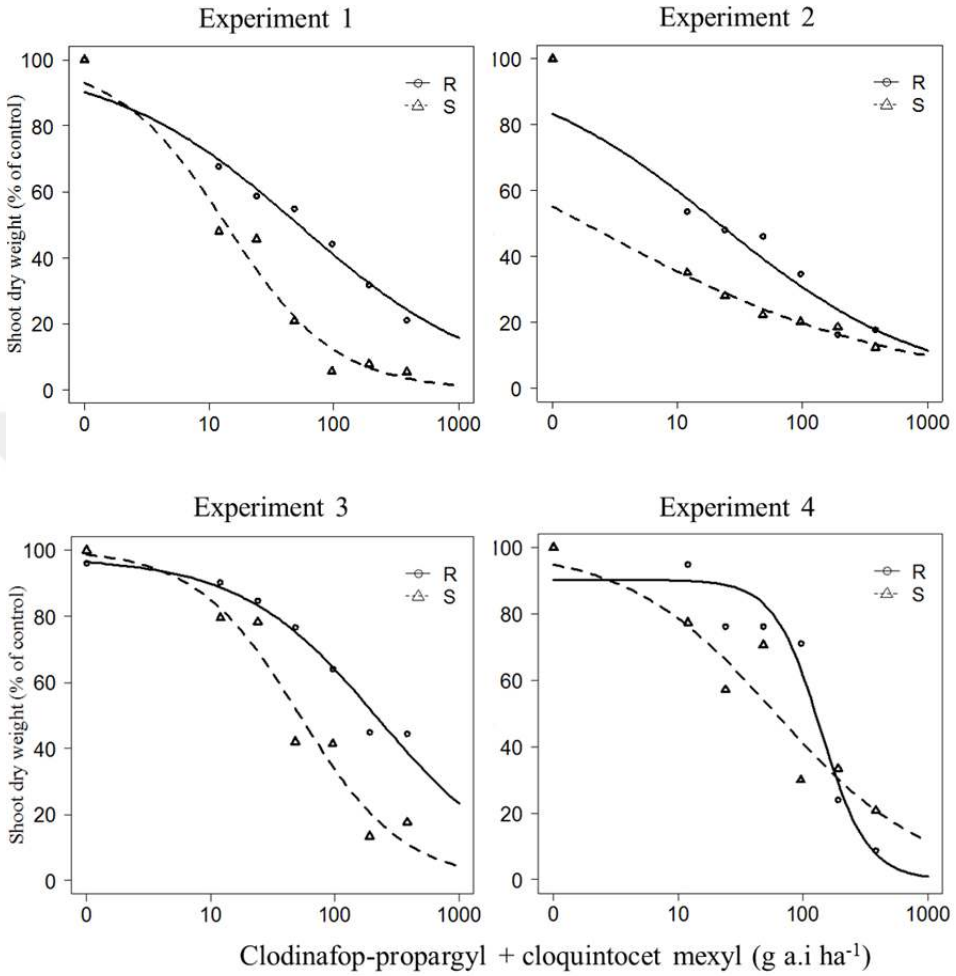

Figure 4.20. Dose-response curves for above-ground shoot dry weight of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of clodinafop-propargyl+cloquintocet-mexyl.

In pinoxaden + cloquintocet-mexyl, the estimated ED_{50} values of S and R populations and RI's values from the four experiments are presented in Table 4.11. The S population was effectively controlled by the recommended field rate of pinoxaden (40.5g a.i ha^{-1}) and partially controlled in R population (Figure 4.21). At the higher dose of pinoxaden (324g a.i ha^{-1}), S and R plants were killed (mean dry

weights ≤ 20%, except for experiment 3 where dry weight was ≥ 20% of untreated controls) (Figure 4.22). Goodness-of-fit curves obtained from experiment 1 and 2 the pinoxaden doses reduced the dry weight of S and R populations dramatically.

Table 4.11. Regression parameters estimated from the greenhouse test based on dry weight (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with pinoxaden+cloquintocet-mexyl

Exp no	Pop	Regression parameter				p-val	R^2	RI
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)			
1	S	-	80.1 (9.1)	0.59 (0.2)	9.14 (5.4)		0.99	
	R	-	80.8 (9.2)	0.49 (0.1)	15.84 (9.4)		0.99	1.73
2	S	-	63.9 (6.7)	0.47 (0.1)	1.70 (2.0)		0.99	
	R	-	59.8 (6.7)	0.40 (0.1)	3.51 (3.1)		0.97	2.06
3	S	19.7 (17.2)	100.8 (9.7)	1.15 (0.6)	30.54 (17.7)		0.99	
	R	26.5 (33.0)	99.3 (10.2)	0.91 (0.7)	51.23(61.4)		0.98	1.67
4	S	-	98.4 (8.0)	1.21 (0.2)	24.52 (5.7)	***	0.98	
	R	-	97.5 (8.2)	1.04 (0.2)	34.34 (9.2)	***	0.99	1.40

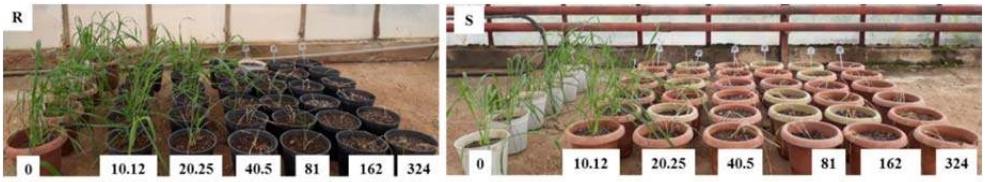

Figure 4.21. Effect of pinoxaden+cloquintocet-mexyl (g a.i ha^{-1}) on resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.), on the 28th day after treatment.

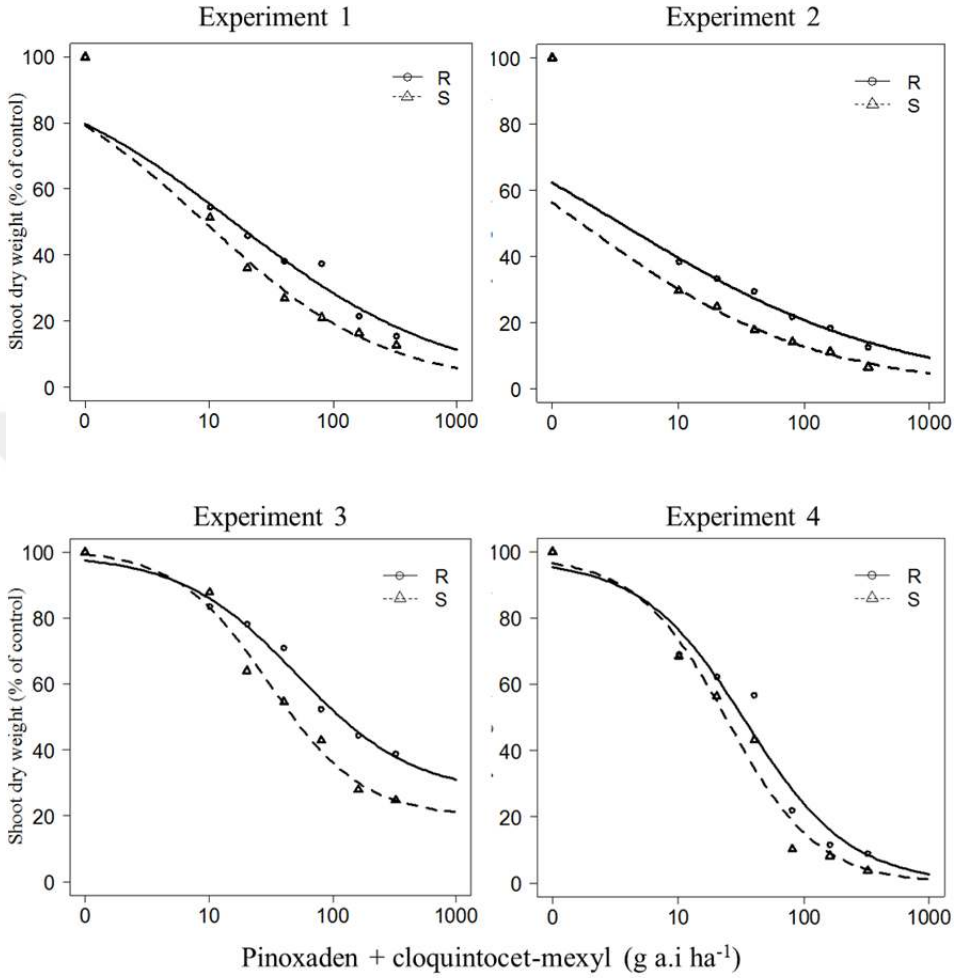

Figure 4.22. Dose-response curves for above-ground shoot dry weight of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of pinoxaden+cloquintocet-mexyl.

For mesosulfuron-methyl+iodosulfuron, estimated ED_{50} values and RI's values for the four experiments were given in Table 4.12. The S plants were killed by the recommended rate of mesosulfuron-methyl + iodosulfuron (9g a.i ha^{-1}), but the R plants were survived 100% in the same dose (Figure 4.23). The dose-response study showed that the reduction in shoot dry weight was decreased with the increased herbicide dose. The decreased dry weight of the S population

occurred at lower doses in four experiments compared to the R population (Figure 4.24)

Table 4.12. Regression parameters estimated from the greenhouse test based on dry weight (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with mesosulfuron-methyl+iodosulfuron

Exp no	Pop	Regression parameters						
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)	*p-val*	R^2	*RI*
1	S	-	99.2 (10.5)	0.86 (0.2)	3.66 (1.5)	*	0.99	
	R	-	80.8 (10.4)	0.35 (0.1)	4.78 (4.2)		0.99	1.30
2	S	-	99.8 (4.9)	1.42 (0.3)	1.50 (0.3)	***	0.99	
	R	-	83.7 (4.9)	0.63 (0.1)	1.63 (0.5)	**	0.97	1.09
3	S	17.7 (4.9)	101.2 (6.1)	2.05 (0.6)	3.75 (0.6)	***	0.99	
	R	5.1 (23.7)	99.7 (6.4)	0.79 (0.3)	9.33 (7.1)		0.98	2.48
4	S	10.5 (15.9)	100.2 (9.1)	1.00 (0.6)	3.38 (1.6)	*	0.98	
	R	18.9 (23.9)	100.3 (8.9)	1.10 (0.6)	10.86 (7.8)		0.99	3.21

Figure 4.23. Effect of mesosulfuron-methyl+iodosulfuron (g a.i ha^{-1}) on resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.), on the 28th day after treatment.

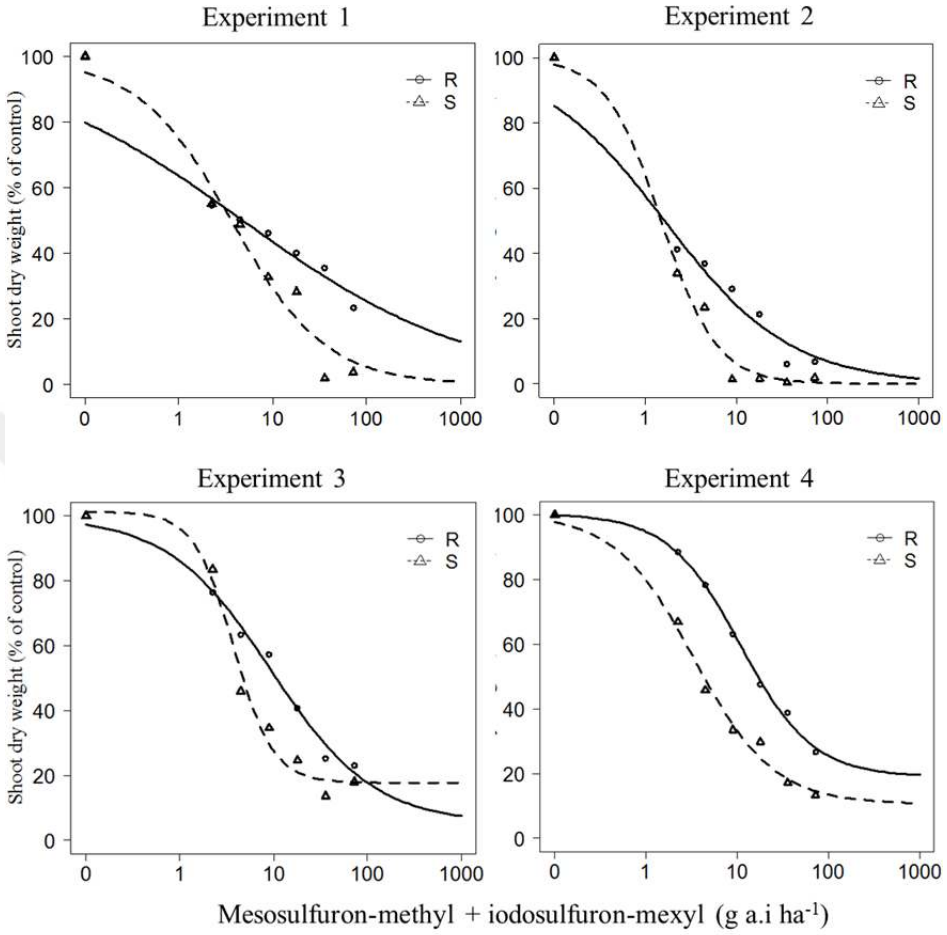

Figure 4.24. Dose-response curves for above-ground shoot dry weight of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with increasing doses of mesosulfuron-methyl+iodosulfuron.

For pyroxsulam+cloquintocet-mexyl, estimated ED_{50} values and RI's values were summarized in Table 4.13. Plants of S and R populations are survived (≥50%) at field rates of 18.75g a.i ha^{-1} pyroxsulam (Figure 4.25). Dose-response study in experiment 1, 2 and 4 a goodness fit was obtained from the log-logistic equation (Figure 4.26), confirmed that ED_{50} of R population in experiment 1 was high significantly more than the S population, resulting in RI's more than 42.18.

Table 4.13. Regression parameters estimated from the greenhouse test based on dry weight (%) of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with pyroxsulam+cloquintocet-mexyl

Exp no	Pop	Regression parameters				p-val	R^2	RI
		c (±SE)	*d* (±SE)	*b* (±SE)	ED_{50} (±SE)			
1	S	-	80.9 (9.9)	0.23 (0.1)	0.34 (1.1)		0.99	
	R	-	43.0 (9.9)	0.43 (0.1)	14.58 (9.3)		0.99	42.18
2	S	-	85.9 (10.5)	0.44 (0.1)	8.24 (6.0)		0.99	
	R	-	78.2 (10.6)	0.56 (0.1)	31.7 (17.2)		0.97	3.84
3	S	15.7 (10.1)	100.1 (6.4)	1.05 (0.4)	8.18 (2.7)	**	0.99	
	R	8.2 (30.2)	100.1 (6.3)	0.83 (0.3)	27.95 (26.2)		0.98	3.41
4	S	-	75.8 (6.5)	0.79 (0.2)	2.57 (1.1)	*	0.98	
	R	-	75.7 (6.5)	0.62 (0.1)	3.74 (1.5)	*	0.99	1.45

*Significant codes: *($p \leq 0.05$), **($p \leq 0.01$), ***($p=0.001$); R^2=coefficient of determination

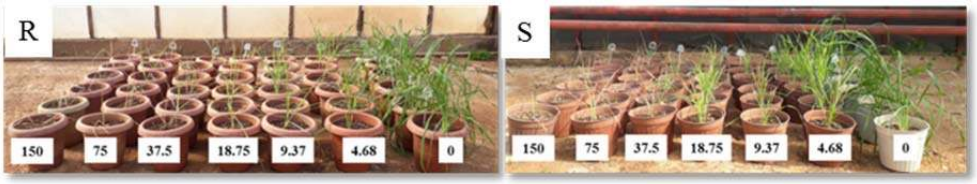

Figure 4.25. Effect of pyroxsulam+cloquintocet-mexyl (g a.i ha^{-1}) on resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.), on the 28th day after treatment.

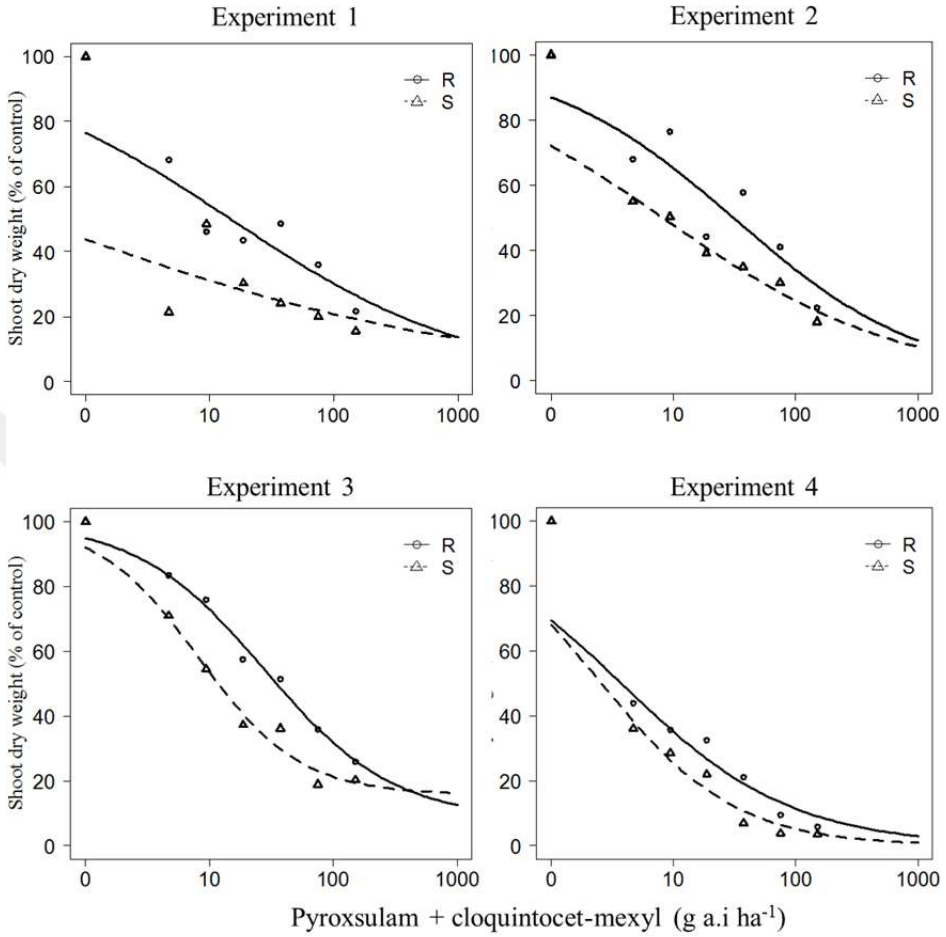

Figure 4.26. Dose-response curves for above-ground shoot dry weight of resistant (R) and susceptible (S) populations of sterile wild oat (*Avena sterilis* L.) treated with doses of pyroxsulam+cloquintocet-mexyl.

4.3. Molecular Test for Detecting Herbicide Resistance in *Avena sterilis* L.

DNA fragments amplified by the primer pairs ACCase-F and ACCase-R resulted in a sequence of 3044 bp in length (Figure 4.27). The nucleotide sequence of R and S populations has been deposited in the gen-bank database, accession numbers are given in Table 4.14 and Table 4.15. The DNA sequence of R and S populations obtained from the primer ACCase-F and ACCase-R was slightly

higher in quality than those obtained from the ALS-F and ALS-R primers (not shown). The total length of the conserved ACCase gene was 1843bp, encompassing the eight known point mutations for ACCase-inhibiting herbicide resistance.

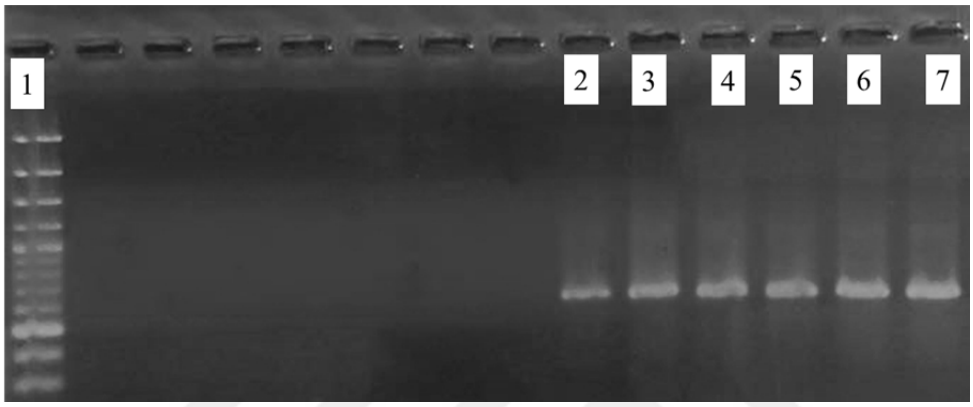

Figure 4.27. Amplicons obtained with primers pairs ACCase-F and ACCase-R.

1- Marker; 2- Untreated resistant plants; 3-Untreated susceptible plants; 4- resistant plants treated with clodinafop-propargyl; 5- susceptible plants treated with clodinafop-propargyl; 6- resistant plants treated with pinoxaden; 7- susceptible plants treated with pinoxaden.

Table 4.14. DNA sequence of resistance (R) and susceptible (S) sterile wild oat (*Avena sterilis* L.) treated with clodinafop-propargyl

Pbiotypes	Accession	Sequence
S	MK534333	ATT TCC CAG TGG CAG GAT TGA TAT TAC TTT TAG ATC ATT TGC TGT TAC CAA CCT
		Gln Phe Gln
R	MK534334	ATT TCC - - G TGG CAG GAT TGA TAT TAC –TT TAG ATC ATT TGC TGT TAC C–A CCT

Table 4.15. DNA sequence of resistance (R) and susceptible (S) sterile wild oat (*Avena sterilis* L.) treated with pinoxaden

biotypes	Accession	Sequence
S	MK534330	ATG GTC GTA TTA TTA TCG ATT CTG TTG CAT ACG AGG TTG GCA TAC GGT GCA TAC
		Val Ser Thr Gly
R	MK534329	ATG G -C GTA TTA TTA T- G ATT CTG TTG CAT A - G AGG TTG GCA TAC G -T GCA TAC

The protein sequences were highly conserved between the R and S populations. In particular, a single amino acid replacement at Glutamine (Gln), Phenylalanine (Phe), Valine (Val), Serine (Ser), Threonine (Thr) and Glycine (Gly) was identified to confer cross-resistance to ACCase-inhibiting herbicide. In a previous study, the mutation of Gly substitution was confirmed in *A. sterilis* populations against ACCase herbicide in Australia (Liu et al., 2007). The Ser substitution was first detected in kochia [*Kochia scoparia* (L.) Schrad.)] treated with chlorsulfuron (Guttieri *et al.*, 1995; Li *et al.*, 2015). Other amino acid substitutions within the ACC protein sequence confirmed resistance in other grass weed species as target-site resistance to ACCase and ALS herbicides include Ile1781Val, Trp1999Leu, Ile2041Val, Cys2088Arg, and Gly2096Ala (Warwick *et al.*, 2010; Beckie *et al.*, 2012b; Li *et al.*, 2015; Saini *et al.*, 2015). The patterns of cross-resistance differed between mutations (Delye *et al.*, 2002a; 2003; 2005; Délye and Michel, 2005). Additionally, sequencing provides reliable, quick results, while the results from ACCase can vary considerably depending on the DNA extraction process or on incubation buffer used (Shukla *et al.*, 1997; Délye and Michel, 2005).

4.4. Comparison of Different Assay Techniques

After repeating the quick assays (agar-based test) with *A. sterilis* population and confirming the results with a greenhouse and filter paper-based test, the agar-based seed germination test seemed more effective. The pros of this method are the quickness of results and the limited space required to conduct the study compared to the greenhouse test, does not require watering or any other plant maintenance and much lower need for working material and human effort (Kashani *et al.*, 2007; Sasanfar *et al.*, 2017; Abdurruhman and Uygur, 2018a). Additional pro in agar-based seed germination test is the opportunity of enhancing germination with gibberellin (Cirujeda *et al.*, 2001). Some disadvantages in agar-based seed germination test was noticed, for instance, measuring the total lengths of the

coleoptile and hypocotyl for germinated seeds (mm or cm) per petri dish has been a time-consuming process and higher dose of herbicide would not prevent the seed germination of susceptible *A. sterilis* population, although growth was stopped after the cotyledon stage (Abdurruhman and Uygur, 2018b), and herbicide treatment is totally different from field applications. Furthermore, seed dormancy issues can affect seed germination in agar medium, as it has been the case for assay beginning directly after seed collection (Moss, 1995; Kaundun *et al.*, 2014).

Greenhouse and filter paper-based tests differ from the agar-based test on preliminary materials such as bench-top, infrastructure and take more than 4 months from seed collection to evaluate resistance (Kuk *et al.*, 2003; Burgos, 2015). In the filter paper-based test, once the seeds are placed on the top of filter paper, they need water regularly until germination, fine sand is also needed to retain plants stand and maintain the moisture. Unexpected challenges of the filter paper test were experienced. For example, plants may bend over due to the limited space (as a Petri-dish), maintenance of moisture without water access and fluctuating temperature or light inside the phytotron or growth cabinet (Abdurruhman and Uygur, 2018a). The estimated lowest ED_{50} of R and S populations for each experiment were summarized in Table 4.16. The greenhouse test revealed that the R population was highly resistant to clodinafop-propargyl (>11-fold) and pyroxsulam (>42-fold) as compared to the S population, indicated that the population had evolved multiple resistance (ACCase and ALS herbicides). Whereas cross-resistance also occurred.

Table 4.16. The lowest ED_{50} values of four herbicides from different resistance tests

	Greenhouse test	Filter paper-based test	Agar-based seed germination test

Herbicides	Pop.	ED_{50}	R/S	ED_{50}	R/S	ED_{50}	R/S
Clodinafop-propargyl	S	1.8		17.46		1.58	
	R	21.56	11.93	78.2	4.47	4.78	3.00
Pinoxaden	S	1.70		25.57		0.39	
	R	3.51	2.06	62.11	2.42	1.17	3.00
Mesosulfuron-methyl	S	1.50		1.40		0.72	
	R	1.63	1.09	4.30	3.05	1.95	2.67
Pyroxsulam	S	0.34		8.16		1.28	
	R	14.58	42.18	34.29	4.20	3.28	2.55

In previous studies, herbicide resistance levels were classified as no resistance (RI<2); low resistance (RI = 2-5); moderate resistance (RI = 6-10); and high resistance (RI >10) (Beckie and Tardif, 2012; Moss *et al.*, 2019). Resistance levels obtained from the agar-based, filter paper-based, and greenhouse tests, differed significantly in among individual plant of the same population. This may be due to differences of resistance levels within the individual plants in the assays or resistance evolved in this population was still in progress (Uludag *et al.*, 2007; Nkoa *et al.*, 2015). Similar variations in resistance levels were observed when laboratory assays were validated with whole-plant pot assay (Moss *et al.*, 1998; Kaundun *et al.*, 2011b). Our findings are in line with Lamego *et al.* (2009) where they reported that the appearance of the highly resistant weed biotypes was attributed to the absence of herbicide rotation and strong selection pressure. Rubin (1996) proposed that resistant weed populations might come to the surface after more than three years of continuous herbicide treatment and it can remain resistant to the herbicide for seven years after application (Keshtkar *et al.*, 2019). Some studies have proved that low rates of a herbicide can result in the development of herbicide resistance (Manalil *et al.*, 2011), Susceptible population of rigid ryegrass was selected by low rates of diclofop resulted in the rapid evolution of resistance (Neve and Powles, 2005a, b). In molecular test when compared to the quick and greenhouse tests, it required less time (approximately 2-5 days), labor, and space than other tests and was simpler (Xu *et al.*, 2010).

5. CONCLUSION

Herbicide-resistant weeds will migrate from fields to fields no matter what crop management practices have been used (Llewellyn *et al.*, 2002). However, herbicide resistance can originate in a field through either gene flow by pollen or seed movement from adjacent areas where herbicide-resistant weeds grow, from natural and independent mutations occurring in weed populations, or from both (Merotto *et al.*, 2010; Michael *et al.*, 2010).

Farmers should monitor weed resistance when using ACCase and ALS inhibitors and herbicide rotation should be a key component of any management strategy to prevent weed resistance.

Results from this study proved the previous work done by Uygur *et al.* (2013) and Uygur *et al.* (2014). Wheat-growing fields in Adana contain sterile wild oat populations with resistance to ACCase and ALS herbicides, as they have been the most commonly utilized to control *A. sterilis* in wheat crops. These herbicides are no-longer effective in controlling *A. sterilis* populations and are likely to be unsuccessful in the near future. The results from the DNA analysis showed that resistant and susceptible populations survived applications of ACCase and ALS revealed many of the same mechanisms of resistance documented in previous research were present in Adana *A. sterilis.* This research is the first documented case of field-evolved target-site resistance to the ACCase herbicides in *Avena sterilis* in Adana.

Looking to the future, it is unlikely that the reversal of herbicide resistance is possible. Managing the current resistance levels in *A. sterilis* is likely the most probable action. The use of multiple effective herbicides with different mechanisms of action along with the integration of cultural and mechanical control practices as well as crop rotation will be vital to manage *A. sterilis* populations in Adana in the future.

We continue to learn about the aspects of weed resistance, we also continue to improve and differentiate the methods for surveying, sampling, and testing for weed resistance. A good field survey, seed sampling, seed storage, choice of test, use of susceptible populations and data analysis are critical for designing weed resistance management or mitigation strategies. However, each step varies depending on numerous factors, mainly the herbicide MoA, weed species, the timing of application, and the test goals. The partnership and communication between university, research institutes, herbicide companies, and farmers are of great importance for the detection of herbicide resistance weed and planning for the management program.

Greenhouse test is still the most commonly used test for determining resistance to herbicides because it is parallel to plant response in the field and is liberated of the mode of action. The results of the agar-based test are rarely compared with those of the greenhouse tests because the herbicide doses in Petri-plate test and agar-based tests are much lower than recommended field doses. Many quick tests are specific to the herbicide mode of action or weed species, molecular-based tests are specific to the target-site resistance mechanism (Table 5.1). Therefore, selecting the appropriate test is critical. Consistency, efficiency, and cost of the test are major considerations for commercialization.

Table 5.1. General advantages and disadvantages of different resistance test methods used in the study

Method	Test duration	Quick result	Suitable for all weed sp.	Mimics field conditions	Suitable for all herbicides	Detects R-mechanism	Cost
Agar-based seed germination test	7-14 days	Yes	No	No	Most	No	Medium
Agar-based seedling test	10-15 days	Yes	No	No	Most	No	Medium
Filter paper-based seed test	2 months	No	No	Yes	Yes	Yes	High
Greenhouse test	3 months	No	Yes	Yes	Yes	Yes	High
Molecular test	2-5 days	Yes	Yes	No	No	No (TSR only)	Very high

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CURRICULUM VITAE

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He received his B.Sc. honors class one in Agricultural Science (Crop Protection option) from the University of Khartoum, Sudan in 2009. He joined the Department of Crop Protection of the University of Khartoum as a teaching assistant in 2010.

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In September 2014, he received a Ph.D. scholarship offered by both the Turkish Government and the Ministry of Higher Education and Scientific Research Sudan. In order to learn Turkish language, he joined Cukurova University Language Center "TÖMER" from October 2014 until July 2015. In September 2015, he was registered as a Ph.D. student in the Department of Plant Protection of Cukurova University.

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