Investigations of Alterations of Membrane Responses to Diclofop as Mechanism of Resistance in Wild Oat

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ABSTRACT

Resistance to graminicide, diclofop-methyl, has recently appeared in wild oat (*Avena fatua* and *A. sterilis*). The effects of diclofop on the plasma membrane potential were investigated in herbicide-resistant wild oat plants. The results indicated that wild oat had no correlation between resistance to graminicides and recovery of the plasma membrane potential. However, the recovery of the plasma membrane potential is pH dependent with a sharp reflection point. Recovery in four biotypes, two resistant and two susceptible, occured at pH's greater than 6.3 to 6.5. The lack of correlation between recovery of the plasma membrane potential following removal of herbicide and herbicide resistance in wild oat suggests rapid recovery of the plama membrane potential is not a pre-requisite for resistance.

Key words: Diclofop, herbicide resistance, membrane potential, wild oat

INTRODUCTION

Aryloxyphenoxypropionate (APP) herbicide, diclofop-methyl, was introduced in the late 1970s for control of grass weeds in both cereal and dicot crops. APP herbicides inhibit the chloroplastic enzyme, acetyl Coenzyme A carboxylase (ACCase), which is the first step in fatty acid biosynthesis. These herbicides only inhibit ACCase from monocot species and are only toxic to grass. In addition, these compounds can depolarize the plasma membrane resulting in the loss of membrane integrity (Shimabukuro 1990). Recently at least seven species of grass weeds have developed resistance to diclofop.

Research on mechanism of APP herbicides resistance in *Setaria viridis* (Marles *et al.* 1993), *Avena sterilis* (Maneechote *et al.* 1994) and *Sorghum halepense* (Marles and Devine 1992, unpublished) has revealed altered forms of ACCase that are much less sensitive to these herbicides. However, membrane depolarization by diclofop has been proposed to be an alternative mechanism of resistance to ACCase-inhibiting herbicides (Shimabukuro and Hoffer 1992). Initially, diclofop was presumed to act as a protonophore in the plasma membrane (Wright and Shimabukuro 1987). Later, this hypothesis was revised by Shimabukuro and Hoffer (1992) who suggested that the collapse of the proton motive force in response to diclofop acid is due to the specific interaction of diclofop with a plasma membrane protein. APP herbicides have the ability to depolarize plasma membrane potentials in a number of susceptible and resistant species (Lucas *et al.* 1984, Wright and Shimabukuro 1987, DiTomaso *et al.* 1991, Häusler *et al.* 1991, Holtum *et al.*, 1993). However, in some diclofop-resistant weed biotypes, repolarization of the plasma membrane potentials occurred following removing herbicide from the treatment solution (Häusler *et al.* 1991, Holtum *et al.* 1991, Shimabukuro and Hoffer 1992, Devine *et al.* 1993). Despite these studies, it is still unclear how membrane repolarization following herbicide removal in the resistant biotypes is related to resistance under field conditions since plasma membrane potentials from resistant crops (e.g. wheat and pea) are also depolarized by diclofop acid (Wright and Shimabukuro 1987, DiTomaso *et al.* 1991, DiTomaso 1993). In addition, a full recovery of membrane polarity has been observed in both susceptible corn and resistant pea root cells following herbicide removal (DiTomaso 1991).

A number of membrane-related phenomena

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have been investigated in resistant *Avena fatua*, *Lolium rigidum* and crop species. It has been found that diclofop-induced depolarization and subsequent repolarization of the membrane potential in susceptible and resistant *L. rigidum* is pH dependent (DiTomaso 1993, Holtum *et al.* 1994). Differences in the ability of susceptible and resistant *L. rigidum* and *A. fatua* roots to acidify the external media were also noted (Häusler *et al.* 1991, Devine *et al.* 1993). The roots of the resistant *L. rigidum* biotypes exhibited a decreased rate of acidification of the external solution relative to the susceptible biotypes (Häusler *et al.* 1991).

In this study, the effects of diclofop acid on depolarization and repolarization of plasma membrane potentials in coleoptiles of five resistant biotypes of wild oat were investigated. In addition, other membrane-related experiments such as the effect of pH on recovery of the plasma membrane potential following removal of diclofop acid and the ability of intact roots of these biotypes to acidify the external solution were also conducted to ascertain whether an altered membrane response is a mechanism of resistance to diclofop in these resistant wild oat biotypes.

MATERIALS AND METHODS

Seeds of susceptible (SAS 2 and SAF 19) and resistant (NAF 8, NAS 6, SAF 16, SAF 34, SAS 1 and WAF 12) biotypes of *Avena* spp. collecting from different states of Australia were used in this study. A coding system was used to classify the wild oat seed samples. The first letter is taken from the state of Australia where the biotype was collected. The following letters are the genus and species of wild oats. The number is the order of collection from each state. For example, biotype SAS 1 represents wild oat from South Australia, *Avena sterilis*, collection No. 1. Biotype WAF 12 represents wild oat from Western Australia, *Avena fatua*, collection No. 12.

Measurement of Plasma Membrane Potentials

Etiolated coleoptiles were collected 3 to 5 d after germination on 0.6% agar. The top 3 mm of the coleoptiles were decapitated and the leaves within the coleoptiles were removed. The epidermis was peeled using fine forceps under Higinbotham's solution (Higinbotham *et al.* 1970). A peeled coleoptile was placed in a plexiglass chamber attached to the stage of a microscope. During the impalement of a cell, the coleoptile was continuously irrigated with Higinbotham's solution, pH adjusted to 5.8, at a flow rate of 2.5 mL min^{-1}.

Micropipettes were pulled from borosilicate glass capillaries, 0.86 mm i.d. with an internal filament (GC150F-10, Clark Electrochemical Instruments, UK) to a tip diameter of about 1 μm. The electrodes, with resistances of between 2 to 15 MΩ, were mounted on a head-stage and holder with a Ag-AgCl electrode. The reference electrode, filled with 1M KCl in 2% (w/v) agar, was placed close to the coleoptile in the bathing solution, but downstream from the measuring electrode. Electrode offsets were nulled before each implement. The membrane potential was measured by inserting a glass microelectrode into a single cell by a micromanipulator. Membrane potentials were monitored using a Neuroprobe Amplifier model 1600 (A-M Systems, USA) connected to a recorder. The initial membrane potential was allowed to stabilize for 5 min before herbicide application. The basal solution was replaced with 50 μM diclofop acid in Higinbotham's solution with the final concentration of 1% (v/v) acetone. After 15 min exposure, the treatment solution was replaced with the basal solution. Ten coleoptiles from resistant and susceptible seedlings were used and the data presented a typical run of each biotype.

Effect of pH on Membrane Response to Diclofop Acid

Etiolated coleoptiles of susceptible and resistant wild oat biotypes were used. The experiments were conducted in the same manner as described above. The basal solution was replaced with 50 μM diclofop acid for 15 min. The herbicidal solution was then replaced with the basal solution buffered by 2.5 mM MES-Tris (pH 5.6, 5.8, 6.0, 6.2, 6.4, 6.5). Five to six etiolated coleoptiles were used for each treatment.

RESULTS

Effect of Diclofop Acid on Plasma Membrane Potential

The response to diclofop acid of plasma membrane potentials from etiolated coleoptiles cells of resistant and susceptible biotypes are shown in Figure 1A-F. Initial membrane potentials in coleoptile cells from susceptible and resistant wild oat were

between-100 and -120 mV. In the presence of 50 μM diclofop acid, rapid depolarization of the membrane potential occurred reaching a minimum potential of between-50 and -70 mV within 15 min. Upon removal of herbicide, membrane potentials from all biotypes remained depolarized (Figure 1A-F). No recovery of plasma membrane potential was evident in any of these resistant wild oat biotypes.

A correlation between recovery of the plasma membrane potential following diclofop acid-induced depolarization and resistance to APP herbicides was reported in some resistant biotypes of *L. rigidum* (Häusler *et al.* 1991, Holtum *et al.* 1991, Shimabukuro and Hoffer 1992, DiTomaso 1993) and *A. fatua* (Devine *et al.*, 1993). These resistant biotypes had the ability to recover from diclofop acid induced depolarization whereas the susceptible biotypes did not. To verify that plasma membrane potential could recover in a resistant *L. rigidum* biotype and to check that (in the same system) resistant wild oat biotypes could not, the experiments were also conducted with *L. rigidum*. In this study, coleoptiles of resistant *L. rigidum* biotype SLR 31 showed a recovery of membrane potentials whereas membrane potentials remained depolarized in the susceptible biotype VLR 1 following removal of diclofop acid from the bathing solution (Figure 2). This result confirms the observations of Holtum *et al.* (1991) and provides additional evidence that this recovery phenomenon does not occur in the resistant wild oat biotypes studies here.

Effects of pH on Plasma Membrane Repolarization

The experiments conducted in previous section were performed in Higinbotham's solution (Higinbotham *et al.* 1970) at pH 5.8. The recovery of membrane polarity following removal of ACCase-inhibiting herbicide has been reported in resistant biotypes of *A. fatua* (Devine *et al.* 1993) and *L. rigidum* (Häusler *et al.*, 1991, Holtum *et al.* 1991, Shimabukuro and Hoffer 1992) at pH 5.7. However, a susceptible *L. rigidum* biotype also showed membrane repolarization upon removal of herbicide when the basal solution was buffered at pH 6.0 (DiTomaso 1993). In this case, pH seems to be an important factor in membrane repolarization. To determine whether pH has an effect on membrane response of wild oat biotypes, the repolarization solution was buffered with 2.5 mM MES, at a range of pH from 5.6 to 6.5. Coleoptile cells of two susceptible and two resistant biotypes were examined. At pH 5.6, none of the biotypes demonstrated any recovery of plasma membrane potential following removal of the herbicide (Figure 3). As the pH increased, partial repolarization of the membrane potential was observed in all biotypes and at pH 6.5 full repolarization of the membrane potential was observed in all biotypes. There were some slight differences between biotypes with biotype NAF 8 showing repolarization of the membrane potential at a lower pH than the other biotypes. This biotype had reached 50% repolarization at pH 6.0 compared with pH 6.2 for biotypes SAF 19 and SAS 1 and at pH 6.3 for biotype SAS 2 (Figure 3).

From the results in Figure 3, it is likely that pH affects the ability of plasma membranes to repolarize following removal of diclofop acid. To confirm this observation, single coleoptiles of three resistant and two susceptible biotypes were bathed in 50 μM diclofop acid for 15 min prior to placement in a herbicide-free solution buffered with 2.5 mM MES at pH 6.5. After 20 min, the same coleoptile was treated a second time with diclofop acid for 15 min and the herbicide was replaced with a buffered solution at pH 5.8. The results indicated that an initial diclofop acid-induced depolarization of the membrane potential was reversible in the herbicide-free solution buffered at pH 6.5 (Figures 4 and 5). The second exposure to diclofop acid caused a similar depolarization to the first, but the membrane potential did not recover in a herbicide-free solution buffered at pH 5.8. Biotype NAF 8 showed a full recovery of membrane depolarization within 20 min when the herbicide solution was replaced with solution buffered at pH 6.5 whereas in the other biotypes recovery of membrane polarity was only 60 to 80% of the initial value.

DISCUSSION

Membrane repolarization following diclofop acid-induced depolarization was observed in the resistant *L. rigidum* biotype SLR 31 but not in the susceptible biotype VLR 1 (Figure 2A-B). Similarly, the ability of the diclofop-resistant biotypes to overcome the effects of diclofop acid on plasma membrane depolarization were reported for resistant biotypes of *A. fatua* (Devine *et al.* 1993) and *L. rigidum* (Häusler *et al.* 1991, Holtum *et al.*, 1991, Shimabukuro and Hoffer 1992, DiTomaso 1993). It has been suggested that this recovery of membrane

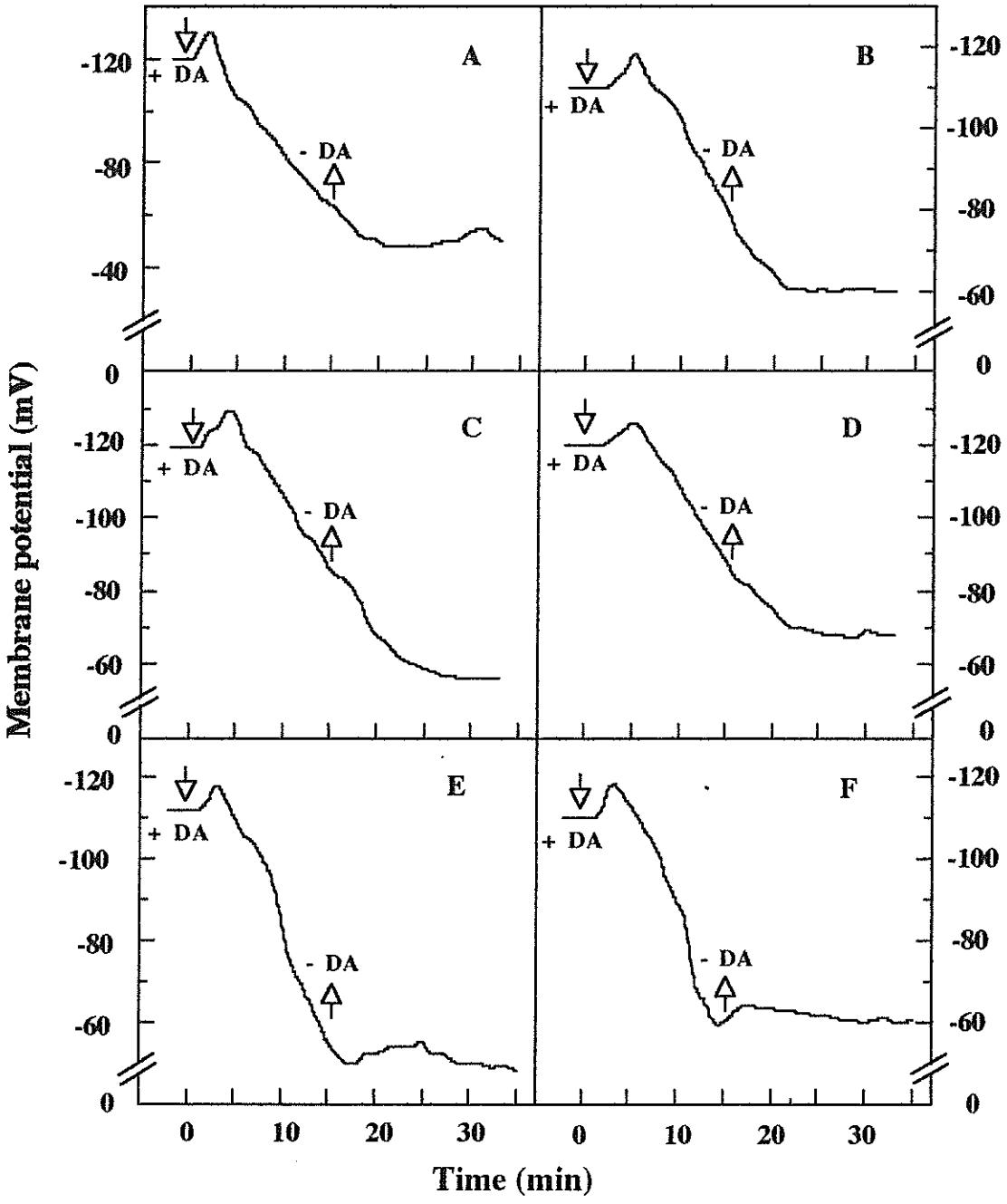

Figure 1.A-F The effect of 50 µM diclofop acid on plasma membrane potentials in coleoptile cells of resistant biotypes NAF 8 (A), SAF 14 (B), SAF 34 (C), WAF 12 (D), NAS 6 (E) and susceptible biotype SAF 19 (F). Arrows, addition (+) and removal (-) of diclofop acid (DA). The data shown is from a single coleoptile and is representative of ten coleoptiles each of the susceptible and resistant biotypes.

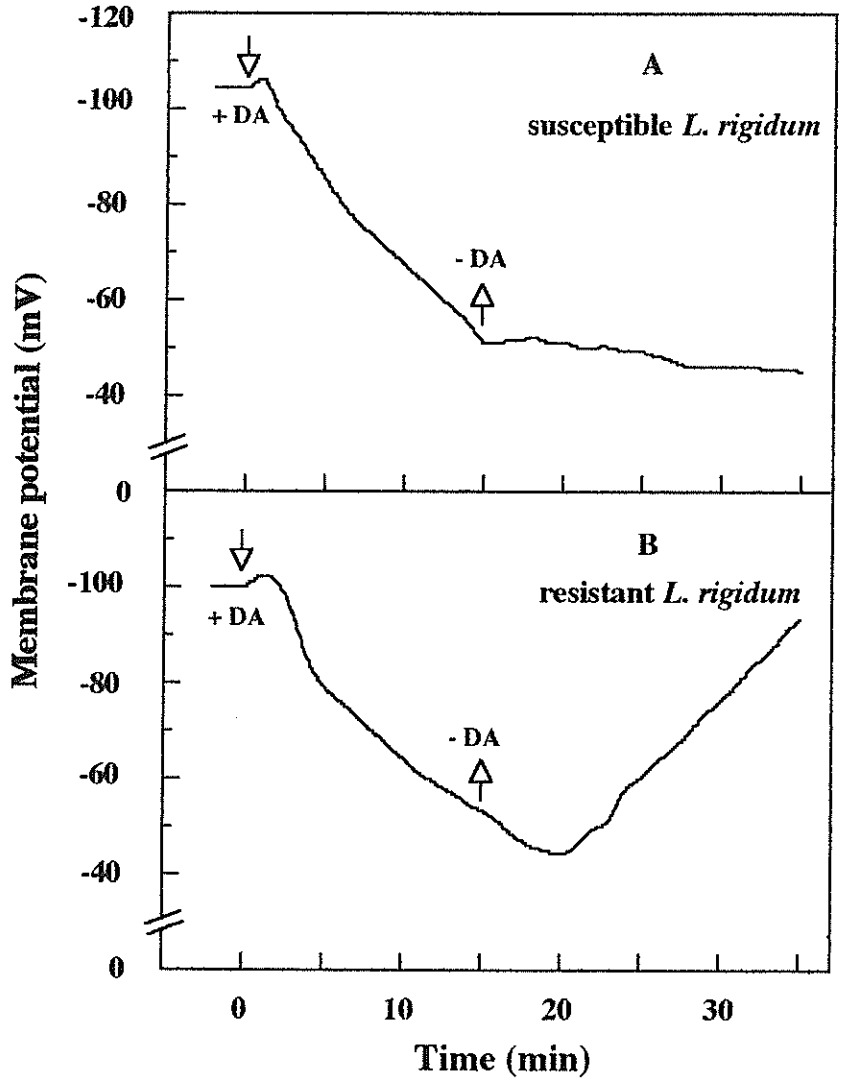

Figure 2.A-B The effect of 50 µM diclofop acid on plasma membrane potentials in coleoptile cells of the susceptible VLR 1 (A) and resistant SLR 31 (B) *L. rigidum*. Arrows, addition (+) and removal (-) of diclofop acid (DA). The data shown is from a single coleoptile and is representative of five coleoptiles each of the susceptible and resistant biotypes.

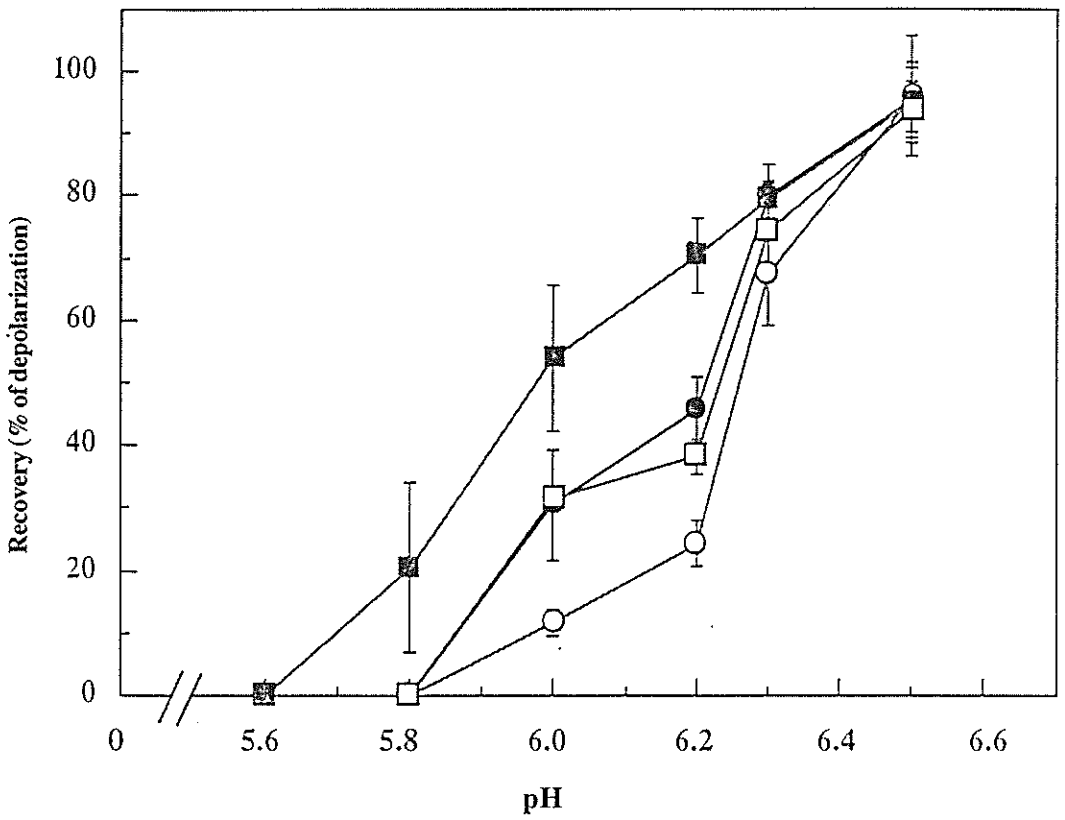

Figure 3. Repolarization of plasma membrane potentials in cells of etiolated coleoptiles of susceptible SAF 19 (□) and SAS 2 (○) and resistant NAF 8 (■), SAS 1 (●) wild oat biotypes as a function of pH of the bathing solution during the recovery phase. Repolarization is presented as a percentage of the full depolarization. Data are the mean ± standard error and calculated on steady-state potentials measured 1 h after the removal of 50 μM diclofop acid.

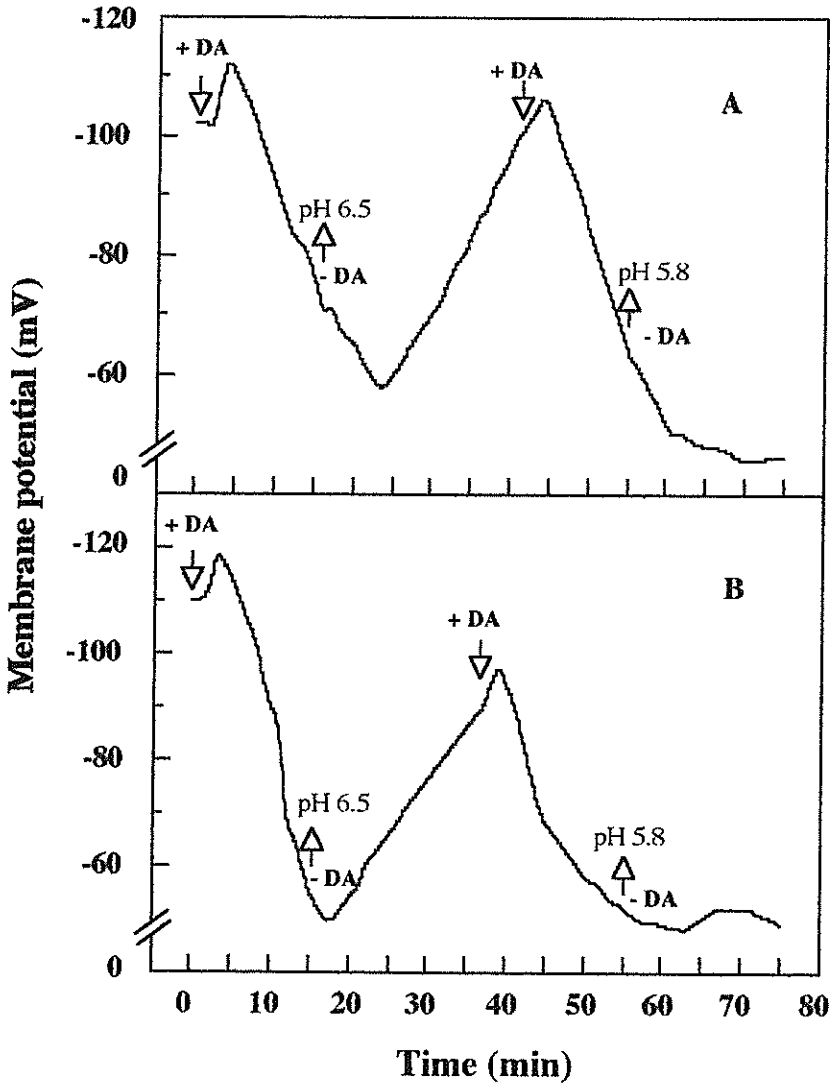

Figure 4.A-B Electrochemical potentials measured in coleoptile cells of the susceptible wild oat biotypes SAF 19 (A) and SAS 2 (B). Arrows, addition (+) and removal (-) of diclofop acid (DA) from the bathing solution. Following removal of diclofop the solution was buffered at either pH 6.5 or pH 5.8. The data shown is a single coleoptile and is representative of five coleoptiles each of the two susceptible wild oat biotypes.

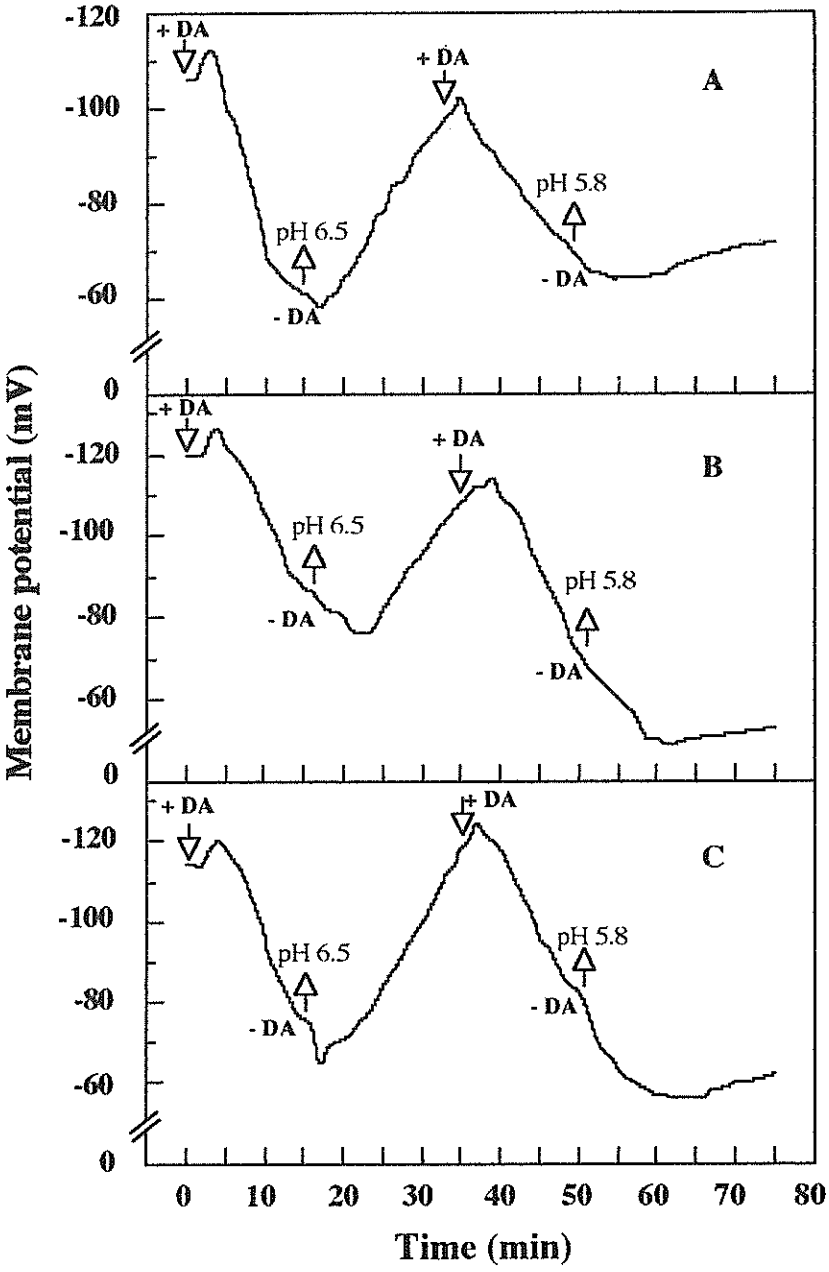

Figure 5.A-C Electrochemical potentials measured in coleoptile cells of three resistant wild oat biotypes NAS 4 (A), SAS 1 (B) and NAF 8 (C). Arrows, addition (+) and removal (-) of diclofop acid (DA) from the bathing solution. Following removal of diclofop acid the solution was buffered at either pH 6.5 or pH 5.8. The data shown is a single coleoptile and is representative of five coleoptiles each of three resistant wild oat biotypes.

polarity following the removal of herbicide is associated with resistance to ACCase-inhibiting herbicides (Häusler *et al.* 1991, Devine *et al.* 1993). This phenomenon did not occur in the resistant wild oat biotypes examined here where the membrane remained depolarized following removal of diclofop acid in both susceptible and resistant biotypes (Figure 1A-E). Clearly, repolarization of the plasma membrane potential following removal of the herbicide in these wild oat biotypes is not correlated with resistance to ACCase-inhibiting herbicides.

The kinetics of depolarization and repolarization of plasma membrane potential depend on the herbicide, the herbicide concentration, the biotype and the pH of the bathing solution (Holtum *et al.* 1994). It is clear from this study and previous reports that pH is an important determinant for the recovery of the plasma membrane potential following removal of diclofop acid (DiTomaso 1993, Holtum *et al.* 1994). It is clear from this study and previous reports that pH is an important determinant for the recovery of the plasma membrane potential following removal of diclofop acid (DiTomaso 1993, Hotum *et al.* 1994). It was proposed that the differences in membrane repolarization following removal of herbicide between susceptible, VLR1, and resistant, SLR 31, biotypes of *L. rigidum* were due to differences in acidification of the cell wall by these biotypes (DiTomaso 1993).

Therefore, strict control of pH by buffer should eliminate the differences between these biotypes. Recovery of membrane polarity is dependent on pH rather than buffering capacity as the resistant biotype SLR 31 will recover from diclofop acid-induced depolarization in a solution buffered at pH 5.8 whereas the susceptible biotype VLR 1 will not (data not shown). In this study the effects of pH on recovery of the membrane potential following removal of diclofop acid were examined (Figure 3) and it is also apparent that different biotypes display different pH profiles for the recovery. Therefore, the differences observed in recovery of the plasma membrane potential between resistant and susceptible biotypes of *L. rigidum* (Häusler *et al.* 1991, Figure 2) and *A. fatua* (Devine *et al.* 1993) might be attributable to different pH profiles for recovery between these biotypes. It has long been known that depolarization of the membrane potential by diclofop acid is pH dependent (Lucas *et al.* 1984, Wright and Shimabukuro 1987, DiTomaso 1993), however, this study demonstrates that recovery from depolarization is also pH dependent (Figure 3). Plasma membrane potentials of coleoptile cells from two susceptible and three resistant wild oat biotypes depolarized by diclofop acid at pH 5.8 recover from depolarization upon herbicide removal if the pH is increased to pH 6.5, but not at pH 5.8 (Figures 4 and 5). Hence, depolarization of the plasma membrane potential by ACCase-inhibiting herbicides is probably not lethal to plants and this phenomenon may be only transitory following field application of these herbicides (DiTomaso *et al.* 1991, DiTomaso 1993).

Two hypotheses have been proposed to explain the diclofop acid-induced depolarization of the plasma membrane potential. Either, that diclofop acid acts as a protonophore (Wright and Shimabukuro 1987), or diclofop acid interacts with a plasma membrane protein to induce an influx of protons (Shimabukuro and Hoffer 1992). Diclofop acid and other ACCase-inhibiting herbicides are weak acids with a pKa of between 3.5 and 4.6 (Dotray *et al.* 1993). At the pH prevalent in the cell wall, pH 5.5 (Sterling 1994), a higher proportion of these herbicides will be in a protonated, lipid soluble form than at the pH of the cytoplasm, pH 7.5. The herbicides will therefore tend to move from the apoplast to the cytoplasm, lose a proton and become trapped in the cytoplasm. This will lead to an influx of protons into the cell and a dissipation of the plasma membrane potential. Increasing the external pH to 6.5 following removal of the herbicide will tend to mobilize some of the protonated diclofop acid from the cell membrane to the external solution where it will be deprotonated. Such a scenario could easily explain the response of repolarization to pH observed in this study (Figure 3). Differences between biotypes in the pH required for recovery of the plasma membrane potential may reflect differences in the amount of diclofop acid which partitions into the plasma membrane or in the microenvironment surrounding the plasma membrane.

If there is a mechanism(s) of resistance which give rise to the repolarization of plasma membrane potential at pH 5.7 in some resistant biotypes of *L. rigidum* (Häusler *et al.* 1991) then it does not operate in the resistant *Avena* biotypes examined here. To date, the molecular basis of repolarization as a resistance mechanism is unknown, but have been postulated to involve sequestration of the herbicide (Holtum 1994). Correlated with this mechanism(s) in *L. rigidum* biotypes is a reduced ability to acidify the external medium (Häusler *et al.* 1991). For example, the roots of susceptible biotypes VLR 1

and SLR 2 decreased the pH of the external medium by 1.7 to 2.4 pH units, whereas the diclofop-resistant biotypes SLR 31, WLR 96, VLR 6 and NLR 2 showed an acidification of less than 0.2 pH units (Häusler *et al.* 1991). DiTomaso (1993) reported that roots of the susceptible biotype VLR 1 were able to acidify the external medium at 4.5 times the rate of the resistant biotype SLR 31. This has been proposed to explain both the membrane repolarization and resistance in one biotype of *L. rigidum*, SLR 31, (DiTomaso 1993). Briefly, this hypothesis suggests that the reduced acidification leads to a higher pH in the apoplast. At this higher pH, more of the herbicide is in the unprotonated form and unable to enter the cell.

In conclusion, the mechanism(s) which give rise to the repolarization of the plasma membrane potential and the re-establishment of acidification by intact roots following diclofop acid treatment in some resistant biotypes of *L. rigidum* and *A. fatua* do not operate in the resistant *Avena* biotypes examined here. Clearly, more work is required to understand these phenomema, however, they are not always correlated with resistance to ACCase-inhibiting herbicides.

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REFERENCES

Devine, M.D., Hall, J.C., Romano, M.L., Marles, M.A.S., Thompson, L.W. and R.H. Shimabukuro. 1993. Diclofop and fenoxaprop resistance in wild oat is associated with an altered effect on the plasma membrane electrogenic potential. Pestic. Biochem. Physiol. 45: 167-177.

DiTomaso, J.M. 1993. Membrane response to diclofop is pH dependent and is regulated by the protonated form of the herbicide in roots of pea and resistant and susceptible rigid ryegrass. Plant Physiol. 102: 1331-1336.

DiTomaso, J.M., Brown, P.H., Stowe, A.E., Linscott, D.L. and L.V. Kochian 1991. Effects of diclofop and diclofop-methyl on membrane potentials in roots of intact oat, maize and pea seedlings. Plant Physiol. 95: 1063-1069.

Dotray, P.A., DiTomaso, J.M., Gronwald, J.W., Wyse, D.L. and L.V. Kochian. 1993. Effects of acetyl-coenzyme A carboxylase inhibitors on root cell transmembrane electric potentials in graminicide-tolerant and -susceptible corn (*Zea mays* L.). Plant Physiol. 103: 919-924.

Häusler, R.E., Holtum, J.A.M. and S.B. Powles 1991. Cross-resistance to herbicides in annual ryegrass *(Lolium rigidum)*. IV. Correlation between membrane effects and resistance to graminicides. Plant Physiol. 97: 1035-1043.

Higinbotham, N., Graves, J.S. and R.F. Davis, R.F. 1970. Evidence for an electrogeniction transport pump in cells of higher plants. J. Membr. Biol. 3: 210-222.

Holtum, J.A.M., Häusler, R.E., Devine, M.D. and S.B. Powles 1994. Recovery of transmembrane potentials in plants resistant to aryloxyphen-oxypropanoate herbicide: a phenomenon awaiting explanation. Weed Sci. 42: 293-301.

Holtum, J.A.M., Matthews, J.M., Häusler, R.E., Liljegren, D.A. and S.B. Powles. 1991. Cross-resistance to herbicides in annual ryegrass *(Lolium rigidum)*. III. On the mechanism of resistance to diclofop-methyl. Plant Physiol. 97: 1026-1034.

Lucas, W.J., Wilson, C. and J.P. Wright. 1984. Perbutation of *Chara* plasmalemma transport function by 2[4 (2', 4'-dichlorophenoxy) phenoxy] propionic acid. Plant Physiol. 74: 61-66.

Maneechote, C., Holtum, J.A.M., Preston, C. and S.B. Powles. 1994. Resistant acetyl CoA carboxylase is a mechanism of herbicide resistance in a biotype of *Avena sterilis* spp. *ludoviciana*. Plant Cell Physiol. 35: 627-635.

Marles, M.A.S., Devine, M.D. and J.C. Hall 1993. Herbicide resistance in *Setaria viridis* conferred by a less sensitive form of acetyl coenzyme A carboxylase. Pestic. Biochem. Physiol. 46: 7-14.

Shimabukuro, R.H. 1990. Selectivity and mode of action of the post emergence herbicide diclofop-methyl. PGRSA Quarterly 18: 37-54.

Shimabukuro, R.H. and B.L. Hoffer. 1992. Effect of diclofop on membrane potentials of herbicide-resistant and -susceptible annual ryegrass root tips. Plant Physiol. 98: 1415-1422.

Sterling, T.M. 1944. Mechanisms of herbicide absorption across plant membranes and accumulation in plant cells. Weed Sci. 42: 263-276.

Wright, J.P. and R.H. Shimabukuro 1987. Effects of diclofop and diclofop-methyl on the membrane potentials of wheat and oat coleoptiles. Plant Physiol. 85: 188-193.