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Mechanism of Paraquat Resistance in *Crassocephalum crepidioides* (Benth.) S. Moore During Immature Stage

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ABSTRAK

Mekanisme kerintangan Crassocephalum crepidioides *terhadap parakuat pada peringkat 6 helai daun dikaji. Parakuat yang diekstrak daripada tisu daun biotip rintang dan rentan tidak mengalami metabolisme. Maka perbezaan metabolisme tidak memainkan peranan dalam mekanisme kerintangan. Biotip rentan menyerap lebih 44% ^{14}C-parakuat berbanding biotip rintang. Namun, lebih daripada 98% ^{14}C-parakuat yang diserap oleh biotip rintang dan rentan berada pada daun yang telah diberikan perlakuan. Perbezaan penyerapan adalah berkolerasi dengan kuantiti lilin epikutikular dan kuantiti membran kutikel pada permukaan daun. Hasil kajian ini mencadangkan bahawa perbezaan penyerapan mungkin merupakan satu faktor yang menyebabkan kerintangan spesies rumpai ini terhadap parakuat.*

ABSTRACT

The mechanism of paraquat resistance in Crassocephalum crepidioides *at the six-leaf stage was investigated. The extractable paraquat was not metabolized by the leaf tissue in the resistant (R) and susceptible (S) biotypes. Therefore, differential metabolism does not appear to play a role in the mechanism of resistance. The S biotype absorbed 44% more ^{14}C-paraquat than the R biotype. However, more than 98% of the absorbed ^{14}C-paraquat remained on the treated leaf of both biotypes. The difference in absorption had a negative correlation with the amount of epicuticular wax as well as the cuticle of leaf surfaces in both biotypes. The results of this study suggest that differential absorption may be a factor that accounts for resistance to paraquat at the six-leaf stage.*

INTRODUCTION

Paraquat is a fast acting, contact herbicide (McEwen and Stepheson 1979) with no activity in the soil due to strong adsorption to soil particles (Kokana and Aylmore 1993). Paraquat is used for controlling a wide range of broad-leaved weeds, sedges and grasses in the rice and vegetable agro-ecosystem for weed clearance before embarking on a new growing season. After the first case of a paraquat-resistant weed (*Conyza bonariensis* (L.) Cronq.) was reported in Egypt in 1979 (Summer 1980), resistance to the herbicide paraquat has been documented for 25 weed species worldwide due to extensive and consecutive use of the herbicide (Heap 1997). However, Chun and Kim have found that *Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. & Mey., a perennial herb belonging to the family Scrophulariaceae, is tolerant to paraquat although this plant has never been sprayed with paraquat (Chun and Kim 1992).

Fuerst and Vaughn (1990) have suggested several hypotheses on the mechanism of paraquat resistance. These include reduced cuticular penetration of paraquat due to differential structure and/or properties of leaf surface, differential metabolism of paraquat, altered site of action, paraquat sequestration from the site of action and enzymatic detoxification of paraquat-generated radical oxygen species. Some of these mechanisms contribute to paraquat resistance in weeds such as *Hordeum glaucum* Steud.

(Bishop *et al.* 1987), *Conyza bonariensis* (L.) Cronq. (Norman *et al.* 1994) and *Erigeron canadensis* (L.) Cronq. (Tanaka *et al.* 1986).

A paraquat-resistant (R) biotype of the annual broadleaf weed *Crassocephalum crepidioides* was first reported in 1990 in tomato fields near Tanah Rata, Cameron Highlands, Malaysia (Itoh *et al.* 1990). *Crassocephalum crepidioides* is also known as redflower ragleaf, a common weed in tomato and potato fields in Cameron Highlands. Paraquat had been applied twice a year at a concentration of 1 kg a.i. ha^{-1} to these fields for ten years. Preliminary studies had shown that the level of resistance in the R biotype of *C. crepidioides* was 100-fold higher than the susceptible (S) biotype through leaf disc tests involving various concentrations of paraquat (Itoh *et al.* 1990). It has been reported that differential translocation was the factor contributing to the mechanism of resistance in mature plants of *C. crepidioides* (Ismail *et al.* 2001). The objective of this study was to compare the mechanism of resistance between the R and S biotypes of *C. crepidioides* at the six-leaf stage.

MATERIALS AND METHODS

Plant Materials

Seeds of the R and S biotypes were collected from the Research Station of the Malaysian Agricultural Research and Development Institute (MARDI) and from farmers' fields in Brinchang, Cameron Highlands, Pahang, Malaysia, respectively. The two populations had been characterized as R and S to paraquat. The seeds were used to establish the six -leaf stage seedlings for the experiments.

Seeds of both biotypes were germinated separately in 36 x 26 x 5 cm plastic trays containing moist sand. After two weeks, seedlings of each biotype were transplanted into 7 cm diameter pots containing sand and grown in the greenhouse at 29±4°C, with a 12-h photoperiod and a light intensity of 800 mE m^{-2} sec^{-1}. Plants in each pot were watered twice daily and fertilized with 5 ml of half strength Hoagland's nutrient solution three times weekly (Hoagland and Arnon 1950).

Herbicide

The herbicide used in the study was paraquat dichloride (Gramoxone) containing 200 g active ingredient (a.i) per litre aqueous solution. Radiolabelled paraquat dichloride ([^{14}C] methyl; specific activity 1.74 x 10^3 kBq mg $^{-1}$) was purchased from Sigma Chemical Co., USA.

Metabolism Study

Four plants of the R and S biotypes at the six-leaf stage were sprayed with nonlabelled paraquat dichloride at 0.50 kg a.i.ha^{-1} using a backpack sprayer that delivered 450 litre ha^{-1} spray volume at 100 kPa pressure. The plants were then treated with 10 µl (1.48 kBq) radiolabeled paraquat in the central portion of the adaxial surface of the third leaf from the plant base. Control vials contained ^{14}C-paraquat with no plant tissue. The treated plants and the control vials were placed in a growth chamber at 25°C in darkness at 65% relative humidity for 12 h. The treated leaf was then excised and the cut petiole end was submerged into a 20-ml vial containing distilled water and then placed in the growth chamber together with the control vial for 12 h at 30°C and light intensity of 50 µE m^{-2} sec^{-1}.

After incubation, the surface herbicide residue was removed by rinsing with 5 ml of distilled water. The radioactive content of the rinse was measured by liquid scintillation spectrometry (LSS) using 10 ml of the aqueous-based cocktail NBCS 104 (Amersham). The tissue was weighed and frozen at -30 ^{0}C until extraction. The frozen tissue was then pulverized with a mortar and pestle using liquid nitrogen. Radiolabelled paraquat was extracted from the tissue with 5 N H_2SO_4 using the fresh weight to volume ratio of 1:4 and centrifuged at 12,000 *g* for 5 min (Chun *et al.* 1997). The resulting supernatant was concentrated with a rotary evaporator at 30°C and analyzed for paraquat and potential metabolites by thin-layer chromatography (TLC) in 5 M NH_4Cl developing system. The TLC plates used were 20 x 20 cm, precoated with silica gel 60 F-254 plates (Merck). Following development, the TLC plate was air-dried at 25°C and placed on X-ray film (Kodak) at -30°C for one month (Craft and Yamaguchi 1964). After the autoradiography study, the TLC plate was separated into 1.5-cm segments from the original to the solvent front, scraped and the radioactivity in each segment was quantified by liquid scintillation spectrometry (LSS) using 1 ml of distilled water and 10 ml of the aqueous-based cocktail NBCS 104 (Amersham). The average of counting efficiency was more than 90%.

Uptake and Translocation Studies

Plants from the two biotypes were sprayed with non-labelled paraquat dichloride at 0.50 kg a.i.ha^{-1} and treated with 10 ml (1.48 kBq) ^{14}C- paraquat dichloride on the third leaf as described previously. The plants were then kept in a growth chamber at 25°C in darkness and 65% relative humidity. The treated leaf was excised at different intervals viz. 0.75, 1.5, 3, 6, 9 and 12 h after incubation. The surface herbicide residue was removed with a 5 ml rinse of distilled water and the radioactive content of the rinse was measured by LSS as described. The excised leaf was then cut into small segments (15-30 mg) and each piece of the segments placed into a pre-weighed 20-ml vial containing 250 µl distilled water, 1 ml NSCII tissue solubilizer (Amersham), 30 µl glacial acetic acid and 300 ml 30% H_2O_2 and incubated for 10 h in a water bath at 50°C (Bishop *et al.* 1987). The vial was cooled at room temperature and 10 ml of the cocktail NBCS 204 was added. It was then kept in the dark for three days before the radioactivity was assessed using LSS. The absorbed paraquat was caculated from the leaf digest and expressed as ng mg^{-1} fresh weight of leaf tissue.

For the translocation study, plants from both biotypes were sprayed with nonlabelled paraquat dichloride and treated with ^{14}C-labelled paraquat dichloride on the third leaf as previously described. Plants were then placed in the growth chamber at 25/30^{0}C night/day temperature and 65% relative humidity. The photoperiod was set at 12 h with a light intensity of 50 µE m^{-2} sec^{-1}. After 24 h incubation, the surface herbicide residue of the treated leaf was removed and the radioactive content of the rinse was determined as before. The whole plant was then exposed to an X-ray film at –30°C for two weeks. After the autoradiography study, the plant was sectioned into three parts: treated leaf, shoot above treated leaf and shoot below treated leaf (including root). The leaf tissue and stem were cut into small segments (15-30 mg) and placed into a preweighed 20-ml vial containing 250 ml distilled water, 1 ml NSCII tissue solubilizer (Amersham), 30 ml glacial acetic acid and 300 ml 30% H_2O_2 and incubated for 10 h in a water bath at 50^{0}C as above. The radioactive content for each part of the plant was quantified using LSS as described before. Translocation of the compound was expressed as a percentage of the total amount absorbed.

Leaf Surface Characteristics Studies

a) Cuticle assay

The third leaves were excised from plants of both biotypes without paraquat application and the total leaf area was measured using a leaf area meter. In order to remove epicuticular wax, the leaves of each biotype were individually submerged in 20 ml chloroform for 5 min. The chloroform was then filtered through Whatman No. 1 filter paper into a pre-weighed, acid-washed and oven-dried beaker. Following the evaporation of chloroform in a fume hood, the wax-containing beaker was allowed to equilibrate to room temperature in a desiccator before being weighed for determining the amount of epicuticular wax (µg cm^{-2}). After the surface wax removal, the cuticle was isolated by digestion of the remaining parts of the leaf tissues in a solution consisting of zinc chloride and hydrochloric acid in a ratio of 2:3 at room temperature for 24 h (Holloway and Baker 1968). Following digestion, the cuticle was extracted from the remaining tissues with 100 ml of methanol. The final methanol rinse (containing cuticular components) was decanted into a pre-weighed, acid-washed and oven-dried beaker. The beaker was dried in a fume hood at room temperature (27ºC) and placed in a desiccator prior to being weighed for determination of cuticle (µg cm^{-2}).

b) Type of stomata, trichome and stomata density determination

The third leaves were excised from untreated plants of the R and S biotypes and fixed individually in a mixture of glacial acetic acid and 70% ethanol (1:3) for 48 h. From the central portion of each leaf blade, a small segment (1.5 x 1.5 cm) was cut, submerged in basic fuschin stain (containing 10% KOH) and placed in an oven at 60^{0}C for one week. The leaf segments were stained in Alcian Green and dehydrated in an ethanol series (50-100%, at 5 min intervals). After dehydration, the specimens were mounted in Euparal. Observations were made on the type of stomata, trichome density (no. cm^{-2}) and stomatal density of the adaxial surface (no. mm^{-2}) using a light microscope (Leitz Diaplan) at 100x magnification.

c) Trichome and epicuticular wax structures

The third leaves without paraquat application were excised from five plants of the R and S biotypes. The central portion of the leaf blades

was cut into small segments (0.5 x 0.5 cm) before fixation in 2% glutaraldehyde for 48 h. Samples were dehydrated in an ethanol series (50-100%, at 5 min intervals), critical-point dried, mounted on aluminium stubs and coated with gold. The structure of trichomes and epicuticular wax of the adaxial surface was observed under a scanning electron microscope (Phillips XL 30).

Statistical Analysis

Each of the above experiments was performed using a randomized complete block design with four replications except for trichome and epicuticular wax structure studies that had three replications. All data were subjected to ANOVA and the means were compared with the t-test at 5% level of significance. A correlation test was undertaken to show the relationship between absorption and leaf surface characteristics in both biotypes.

RESULTS AND DISCUSSION

Metabolism Study

The extraction of paraquat residues from leaf tissues of both biotypes was limited; radioactivity recovered in H_2SO_4 extract accounted for approximately 50 % of the absorbed radioactivity by both *C. crepidioides* biotypes whilst the rest of ^{14}C was unextractable. It is possible that the unextractable radiolabel may have covalently bonded to the leaf tissue (Fuerst and Vaughn 1990). The average of total recovery of the radiolabel, however, was more than 90% of that applied.

Autoradiographs of the standard ^{14}C-paraquat, together with the R and S plant leaf extracts indicated a single spot of radioactivity (R_f = 0.00-0.20). However, radioactivity recovered from acid extracts was not only detected at R_f values of 0.00-0.20 (98.5-99%), but a small amount of radioactivity also appeared at R_f values of 0.20-0.30 and 0.70-0.90 after the TLC plate was quantified using LSS (Table 1). This could be due to the lower sensitivity of the X-ray film to radioactivity as compared to LSS. The large amount (98.5-99%) of radioactivity recovered with R_f values of 0.00-0.20 in the TLC plate corresponded to authentic paraquat while the rest of the radioactivity at R_f values of 0.20-0.30 and 0.70-0.90 were unidentified compounds (Slade 1966). These results suggested that extractable paraquat was not metabolized in the leaf tissues of both biotypes and the differential metabolism could not account for the resistance mechanism of *C. crepidioides* to paraquat as reported in paraquat-resistant weeds like *Conyza bonariensis* (L.) Cronq. (Norman *et al.* 1993) and cultivars like *Lolium perenne* L. (Harvey *et al.* 1978).

Uptake and Translocation Studies

Differential translocation may be due to the difference in degradation at the site of absorption yielding some metabolites that could be translocated and other metabolites that could not be translocated. However, the results from the metabolism study showed that 94-99% of the radioactivity recovered from treated leaf tissue corresponded to authentic paraquat (Table 1). Hence, radioactivity observed in translocation as well as absorption studies can be assumed as authentic paraquat.

Fig. 1 shows the absorbed ^{14}C-paraquat in treated leaves of both biotypes at the six-leaf stage. There was significant difference in its uptake by the R and S biotypes at each time interval. At first, the uptake rate of the S biotype was slower compared to the R biotype. However, the uptake of labelled paraquat by the S biotype increased 3 h after treatment (HAT) and overtook the R biotype approximately 4 HAT. Subsequently, the total uptake by both biotypes plateaued at 6 HAT, indicating that absorption had reached a maximum. There are several barriers to herbicide absorption into the leaf tissue. The first barrier is the trichome, followed by the epicuticular wax and the cuticle. The susceptible biotype absorbed less paraquat compared to the R biotype 3 HAT because it was inhibited by higher trichome density relative to the R biotype (Table 3). Once paraquat passed through the trichomes, the uptake in the S biotype increased and overtook the R biotype approximately at 4 HAT due to a lower amount of epicuticular wax as well as cuticle compared to the R biotype (Table 3). The S biotype absorbed 44% more ^{14}C-paraquat than the R biotype at 6 HAT, suggesting lower total uptake in the R biotype as a factor contributing to paraquat resistance. This result is in agreement with a previous report on *Erigeron philadelphicus* and *E. canadensis* (Tanaka *et al.* 1986).

However, differential translocation does not appear to play a role in the mechanism of resistance because most of the absorbed ^{14}C-

TABLE 1
TLC analyses of radioactivity in acid extracts from leaf tissue of resistant and susceptible biotypes treated with [^{14}C]-paraquat dichloride for 24 h at six-leaf stage

	Distribution (R_f) of radioactivity (% of recovery)		
R_f value	0.00-0.20	0.20-030	0.70-0.90
Standard ^{14}C-paraquat	94 (0.5)	6 (0.4)	*-
R	98.5 (0.4)	-	1.5 (0.1)
S	99 (0.3)	-	1 (0.1)

SE of means are given in parentheses.
* Not detected.

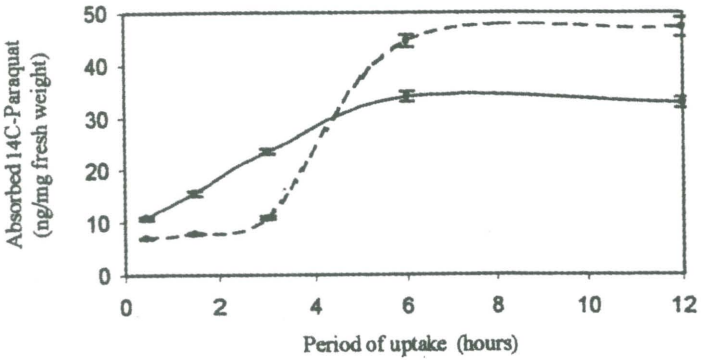

Fig.1: Absorption of ^{14}C-paraquat by leaves of resistant (_) and susceptible (....) biotypes at six leaf stage

TABLE 2
Distribution of total ^{14}C-paraquat recovered in plant leaf in resistant and susceptible biotypes 24 HAT at six-leaf stage

	Distribution of ^{14}C-paraquat (% of absorbed)		
^{14}C-Paraquat in plant sections	Treated leaf	Above treated leaf	Below treated leaf
R	98.99 a	0.78 a	0.23 a
S	98.72 a	1.00 a	0.27 a

Mean within columns with similar letters are not significant at the 5% level by T-test.

paraquat remained in the treated leaves of both biotypes 12 HAT, while less than 2% of the absorbed ^{14}C-paraquat was translocated out of the treated leaves at 24 HAT (Table 2). Qualitative detection of ^{14}C-paraquat distribution in both biotypes using autoradiography confirmed the quantitative results obtained with radioassays (*Fig. 1*). Autoradiographs showed that the absorbed ^{14}C-paraquat still remained in the treated leaf at the site of herbicide application and the adjacent cells in both biotypes.

Nevertheless, further studies are needed to examine the absorption as well as translocation patterns of the R and S biotypes at different temperature conditions and light intensities since the studies were carried out under a single

controlled environment only. Studies have shown that resistance to paraquat in *Hordeum glaucum* (Lasat *et al.* 1996) and *Hordeum leporinum* Link (Purba *et al.* 1995) is temperature-dependent.

Leaf Surface Characteristics Studies

Epicuticular wax and amount of cuticle, stomata and trichome densities in adaxial leaves of both biotypes were studied to observe the relationship between the leaf surface characteristics and paraquat uptake.

Cuticle Content

Several studies have shown that young leaves are more permeable than mature leaves (Leon and Bukovac 1978). This may be due to a change in epicuticular wax composition (Loomis and Schiefertein 1959) during development or that the cuticle from young leaves was not fully developed (Price 1982). Hence, leaves of the same age were used to ensure that the differences observed in cuticle content between the R and S biotypes were not due to the difference in leaf age.

Environment factors that influence the amount of cuticle in a plant include light (Price 1982), temperature (Hull 1958) and humidity (Daly 1964). Greenhouse-grown plants have been shown to have much thinner cuticle compared to field-grown plants (Hull 1958). However, the difference in cuticle content between the R and S biotypes in the present study was not due to environmental factors since both biotypes were planted under the same conditions.

Table 3 shows leaf surface characteristics of *C. crepidioides*. Epicuticular wax of the R biotype was approximately 150% more than the S biotype. The R biotype also had about 50% more cuticle than the S biotype. These results indicate that the third leaf was more difficult to penetrate as the R biotype absorbed 44% less ^{14}C-paraquat compared to the S biotype (*Fig. 1*). Correlation tests also showed that there is an inverse relationship in paraquat uptake with the content of epicuticular wax ($r = -0.95$) and the amount of cuticle ($r = -0.94$). The difference in absorption between the R and S biotypes is correlated to the amount of epicuticular wax as well as the cuticle of the leaf surface, which thereby played a role in the resistance of *C. crepidioides* to paraquat. Pereira *et al.* (1971) reported that nitrofen selectivity in cabbage was due to differences in the cuticular wax. The tolerant cultivar seemed to have thicker cuticle compared to the susceptible cultivar.

Stomata and Trichome Densities

Stomata and trichome densities are two factors that affect herbicide uptake. Guard cells can absorb more foliar-applied herbicides than other epidermal cells because of thinner cuticle (Hull 1970) and/or abundant ecotodesmata (Franke 1961). A thick mat of trichome is known to inhibit the penetration of the herbicide spray; on the other hand, each trichome may serve as portals of entry for spray solution due to thinner walls or less cuticularization near its base (Franke 1967). In this study, however, there was no correlation ($p>0.05$) between stomata, trichome density and paraquat absorption at 6 HAT although there was significant difference ($p>0.05$) in trichome density between the two biotypes (Table 3).

Stomata, Trichome and Epicuticular Wax Structures

Figs. 3, 4 and *5* show the structure of *stomata*, trichomes and epicuticular wax of *C. crepidioides* respectively. These leaf characteristics do not appear to play any role in the mechanism of paraquat resistance since both biotypes are

TABLE 3
Leaf surface characteristics of *C. crepidioides* at six-leaf stage

	R biotype	S biotype
Cuticle (mg cm^{-2})	27.8 a (0.4)	17.9 b (0.3)
Epicuticular wax (mg cm^{-2})	5.3 a (0.2)	2.1 b (0.1)
Stomata (no.mm^{-2})	13.6 a (0.7)	14.6 a (0.8)
Trichomes (no. cm^{-2})	22 a (5)	46 b (4)

Means within rows with similar letters are not significant at the 5% level by T-test. SE of means are given in parentheses.

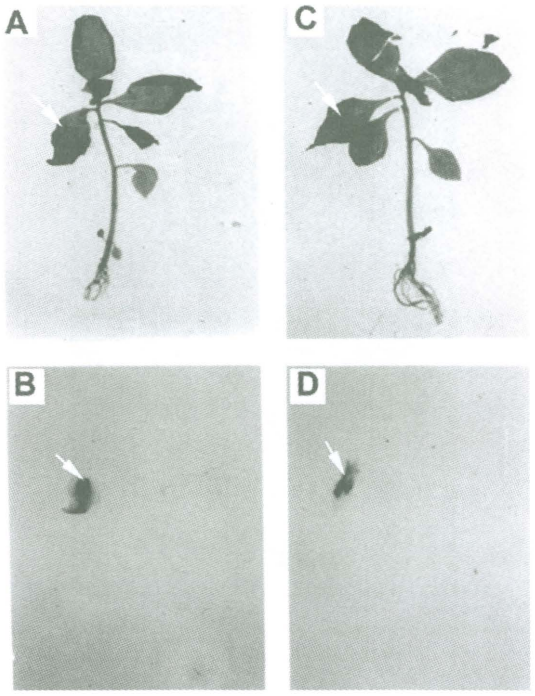

Fig. 2: Distribution of ^{14}C-paraquat in C. crepidioides at six-leaf stage. Photograph (A) and autoradiograph (B) of susceptible biotype; Photograph (C) and autoradiograph (D) of resistant biotype. Arrows indicate site of application

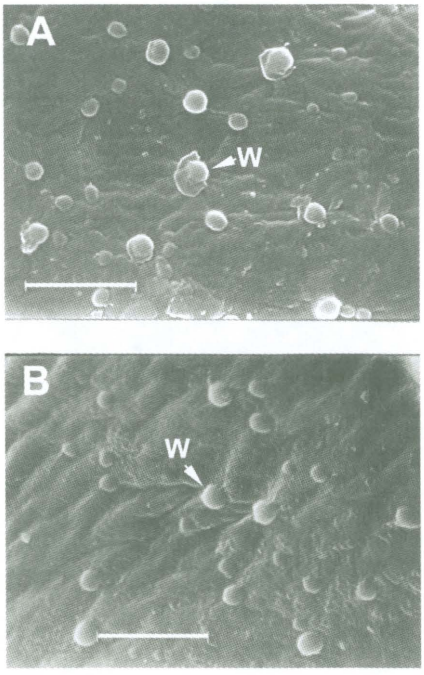

Fig. 3: Anomocytic stoma (S) on adaxial leaf surface of C. crepidioides A) Resistant biotype, B) Susceptible biotype

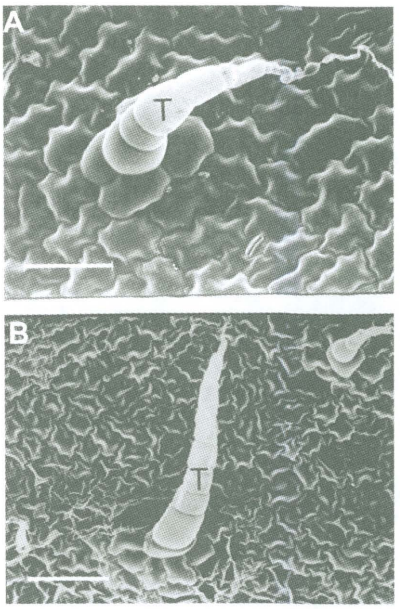

Fig. 4: Multicellular trichome (T) on adaxial leaf surface of C. crepidioides A) Resistant biotype, B) Susceptible biotype

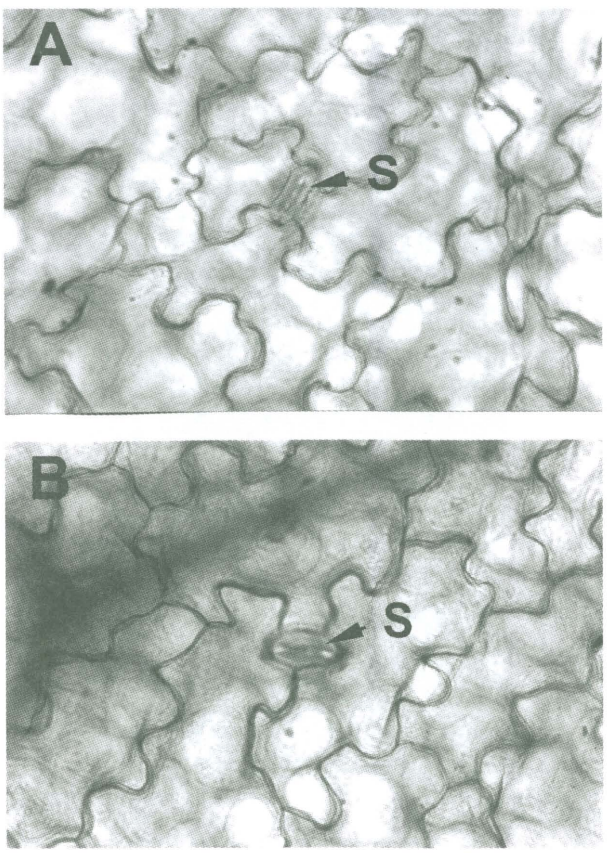

Fig. 5: Granular epicuticular wax (W) on adaxial leaf surface of C. crepidioides A) Resistant biotype, B) Susceptible biotype

identical in these characters. Both biotypes have anomocytic stomata, simple mutlicellular trichomes as well as granular epicuticular wax.

CONCLUSION

It is suggested that in *C. crepidioides*, difference in absorption is a contributing factor for resistance to paraquat at the six-leaf stage. However, further studies are required to see whether resistance is still expressed in changing temperature conditions, light intensities and humidity.

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