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Elemental Changes in Barley Leaves After Infection by *Rhynchosporium secalis*¶

B. SORKHILALEHLOO* and J.P. TEWARI†
Cereals Research Department, Seed and Plant Improvement Institute, Karaj, Iran
E-mail: behzad@ualberta.ca; bsorkhi@yahoo.com

The barley-scald pathosystem was investigated to determine the elemental changes in barley leaves. The potential role of calcium, potassium, silicon and sodium as disease related elements were studied. Energy dispersive X-ray microanalysis (XRM) in conjunction with scanning electron microscopy (SEM) was used to determine elemental changes associated with disease resistance. SEM of barley leaf surfaces inoculated with *Rhynchosporium secalis* showed various shapes of Ca/K-containing crystals associated with plant cells upon infection. Analysis of Ca, Si, K and Na in the superficial layers of plant tissues showed that the levels of these elements changed significantly after inoculation. The extent of changes was dependent on the barley genotype and interactions with scald isolate(s). Calcium and potassium were commonly detected at high levels in the crystals/druses observed in slow-scalding lines, Zavila and UNA 80 similar to those observed in the susceptible cultivar Stander.

Key Words: Barley, Elemental analysis, *Rhynchosporium secalis*, SEM, X-ray microanalysis.

INTRODUCTION

The biochemistry of interaction between *Rhynchosporium secalis* (Oud.) J. J. Davis, the causal agent of the scald disease, and its host, barley (*Hordeum vulgare* L.), has been investigated by a few researchers[1-3]. The barley-scald pathosystem has been investigated to determine the chemical and enzymatic factors involved in the pathogenesis of *R. secalis* and the resistance mechanisms of the host[1,4-6]. Tewari[6] investigated the potential role of calcium as a resistance-related factor and demonstrated that the fungus sequesters calcium from the host tissue during pathogenesis by production of different organic acids. Energy dispersive X-ray microanalyses (XRM) in conjunction with scanning electron microscopy (SEM) have been shown to be useful methods to determine elemental changes associated with disease resistance[7,8]. Silicon has been shown to benefit plants in a number of ways including normal growth and development and through increasing resistance to fungi and other parasites[9,10].

†Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB T6G 2P5, Canada.

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Silicon has been found to be present mainly in the epidermal cell walls, middle lamellae and intercellular spaces within the sub epidermal tissues[10]. Several activities as a pre-existing or dynamic defense resistance factor and its contribution to increase mechanical resistance of injured tissue by silisification have been alluded[9-11]. Silicon is mobilized at the points of penetration by *R. secalis* on barley and the areas enriched in silicon are larger in the resistant than in the susceptible cv.[12]. Calcium ion is considered to be a component of signaling pathways and sodium and potassium ions are directly involved in regulation of intracellular osmotic pressures[13-15]. In recent years, it has become evident that mineral distribution in barley leaves is highly heterogeneous[15]. However, there has been only a little microanalytical work done on the barley-scald pathosystem.

Accumulation of calcium ion in the epidermal cells has been reported to be associated with the formation of halos around the points of penetration of *R. secalis*[16]. Mukerji and Tewari[16] suggested that calcium mobilization at the points of challenge by the pathogen might play a role in host resistance in the barley-scald pathosystem. Tewari[6] detected different calcium-containing crystals which were associated with mobilization of calcium in resistant barley tissues upon challenge by *R. secalis*. While recent work on the barley-scald pathosystem indicates that the hypersensitive response does not occur in incompatible interactions with *R. secalis*[17], the formation of small dark-brown patches on the leaves where infection has been unsuccessful, was referred to as this type of resistance[18].

The current study was undertaken to determine the levels of Ca, K, Si, and Na in barley leaves before and after inoculation with *R. secalis* in both compatible and incompatible interactions using SEM in conjunction with energy-dispersive X-ray microanalyses (XRM) and to characterize disease/resistance-related factors such as crystal formation.

EXPERIMENTAL

Plant materials and inoculation: One scald susceptible barley cv., Stander, two slow-scalding lines, Zavila and UNA 80 and one resistant differential, Osiris, were grown under indoor conditions (at 15 ºC in the dark and then for 2 wks at 18/15 ± 1 ºC day/night temperature under a sealed transparent plastic cover) suitable for evaluation of reactions of seedling to scald. Seven different scald isolates (namely Iso 5, Iso 7, Iso 11, Iso 13, Iso 14, Iso 18 and Iso 19 belonging to Tewari's (co author of this article) barley pathogen collection at the University of Alberta) with known differential reactions on standard resistant cultivars/lines were used to artificially inoculate the seedlings at the 2-leaf stage. Pathogenicity of the isolates used and disease reactions for the selected genotypes were reported by Sorkhilalehloo and Tewari[19]. The final concentration of inoculum was adjusted to 1×10^5 conidia/mL. For disease assessment, seedlings were scored at the 4-leaf stage according to the 0-4 scale, where scores ≥ 2.5 represented susceptible reactions[19].

SEM and XRM: The samples were examined using SEM (Hitachi S-2500, Tokyo) and XRM (JSM 6301-F equipped with IMIX-PST, PGT, Princeton, NJ). The SEM samples were prepared using a standard protocol similar to that explained by Green *et al.*[20]. The samples (4-5 mm^2) were fixed in 2.5 % glutaraldehyde in cacodylate buffer for 1 h followed by 3 washings with the same buffer. The tissues were then post-fixed in 2 % (v/v) osmium tetroxide in cacodylate buffer for 3 h. Following a further washing in double distilled water, the leaf pieces were dehydrated in an ethanol-propylene oxide series. The preparation of samples for the infection study was done using a critical point drying procedure where a critical point drying at 31 ºC was applied to the samples for 10 min. In contrast, the samples for XRM were initially air-dried at 25 ºC for 1 week. Prior to analyzing the plant materials with SEM/XRM, the samples were mounted onto stubs using a double-sided carbon tape, and then sputter coated with gold (model Semprep 2, Nanotech, Cambridge). Using a Link eXL energy-dispersive X-ray system with a light element detector, Ca, Si, K and Na levels at the surface layers of the epidermis of different barley leaf samples were studied at a magnification of X100 and an accelerating voltage of 20 keV.

Statistical analysis: The levels of Ca, K, Si and Na as the percentage of gold used as coating on the surface of leaf samples were used to compare the elemental changes in the barley genotypes with different levels of resistance to the scald pathogen before and after inoculation with various isolates of *R. secalis*. The X-ray microanalysis data were statistically analyzed using a factorial experiment where Iso × Gen interaction was of particular interest. Duncan's multiple means and Tukey's pairwise LS-mean tests were used to check the significance of differences among genotypes/isolates for the traits studied. For the isolates Iso 5, Iso 11 and Iso 13, analyses of variance for the infection and XRM studies were performed using Proc GLM[21].

RESULTS AND DISCUSSION

Scanning electron micrographs of barley leaf surfaces inoculated with *R. secalis* showed various shapes of Ca/K-containing crystals associated with the reactions of plant cells upon infection. Fig. 1 shows crystals formed on top of subcuticular mycelia. Plate-like crystals and druses thereof containing K/Ca (Stander × Iso 13: susceptible reaction), raphide druses containing Ca (UNA 80 × Iso 19: susceptible reaction), druses containing Ca/K (Zavila × Iso 7: susceptible reaction), and bipyramidal crystals containing Ca/Si (Osiris × Iso 13: resistant reaction) were observed. Fig. 2A-D show the XRM spectra for crystals shown in Fig. 1A-D. The dominant peak in Fig. 2A is for K and other notable features are peaks for Ca and Si where druses were found on top of the subcuticular mycelia growing on the leaf surface of Stander. Silicon detected by XRM might also be generated from underneath of the epidermal cell wall. Calcium was commonly detected at very high levels in slow scalding lines (Fig. 1B-C) where druses containing Ca/P and Ca/K were found in compatible reactions of UNA 80 and Zavila (Fig. 2B-C), respectively. Microanalysis

of the crystals observed in Fig. 1D indicated high levels of Ca and Si in the epidermis of Osiris in its resistant reaction to *R. secalis*.

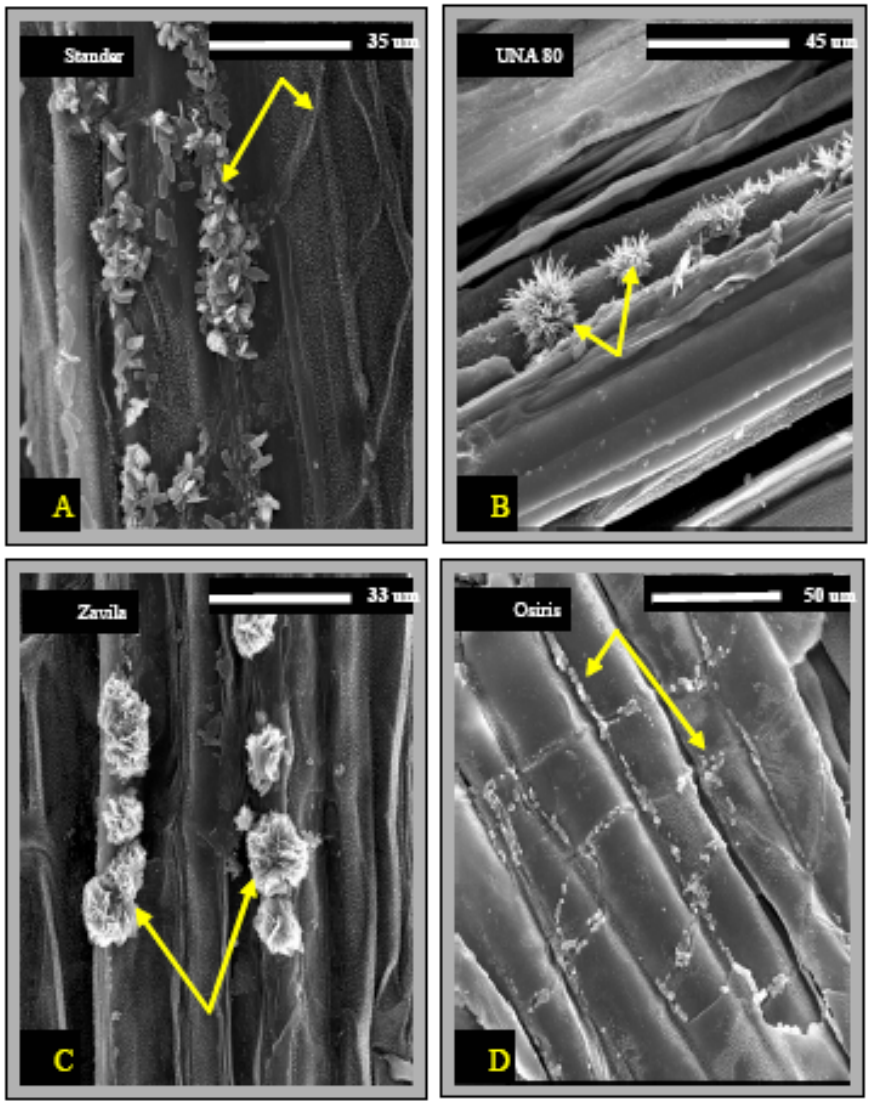

Fig. 1. Scanning electron micrographs of crystals (as shown by arrows) formed on the surface of barley leaves after inoculation with *Rhynchosporium secalis*: (A) Plate like crystals and druses thereof containing potassium/calcium (Stander × Iso 13); (B) Bipyramidal crystals containing calcium/silicon (UNA 80 × Iso 19); (C) Druses containing calcium/potassium (Zavila × Iso 7); and (D) Raphide druses containing calcium (Osiris × Iso 13). Note that all crystals were formed on top of the subcuticular mycelia as a result of after-inoculation responses to the development of fungal hyphae in the plant cuticle

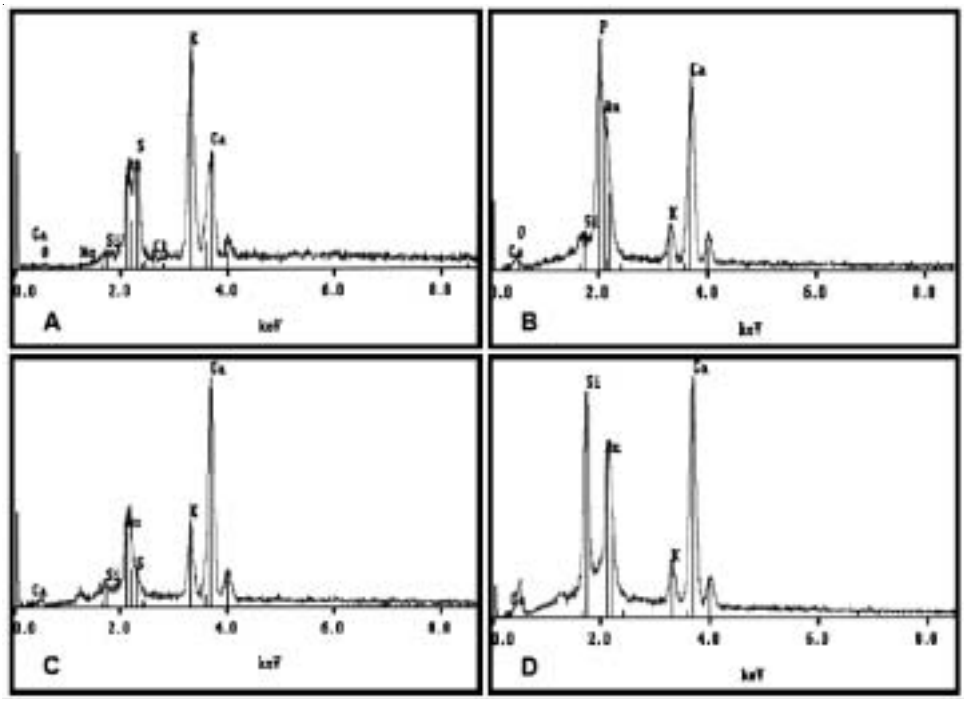

Fig. 2. Energy-dispersive X-ray microanalysis spectra of crystals formed on the surface of barley leaves after inoculation with *Rhynchosporium secalis* different isolates (iso 7, iso 13, and iso 19): A) Plate-like crystals and druses thereof containing potassium/calcium in the susceptible reaction of Stander × Iso 13; (B) Raphide druses containing calcium in the susceptible reaction of UNA 80 × Iso 19; (C) Druses containing calcium/potassium in the susceptible reaction of Zavila × Iso 7; and (D) Bipyramidal crystals containing calcium/silicon in the resistant reaction of Osiris × Iso 13

The XRM data were analyzed to reveal the effects of genotypes, isolates and Gen × Iso interaction. There were significant differences among the genotypes in terms of the elements studied (Table-1). Comparing the average level of elements studied in Stander as a susceptible check with those of the other genotypes, Osiris as the most resistant cultivar showed higher levels of K and Ca. The highest levels of Na were detected for UNA 80 and Osiris. Osiris also showed the minimum level of Si at the surface of epidermis whereas Si for the rest of barley genotypes was not significantly different. Zavila and UNA 80 showed different levels of K and Na, whereas their levels of Ca and Si were not significantly different. Stander had similar K and Na levels as those of Zavila.

Table-2 shows means and groupings for Ca, K, Si and Na levels of leaf samples tested for the inoculation treatments. The healthy samples (the un-inoculated treatment, NI) showed statistically lowest levels of K, Ca, and Si within each genotype.

TABLE-1
CHANGES IN CALCIUM, POTASSIUM, SILICON AND SODIUM LEVELS OF THE LEAF SAMPLES AS % OF GOLD USED AS COATING ON THE SURFACE OF LEAF SAMPLES TESTED FOR THE BARLEY GENOTYPES STUDIED

Genotype	Man as % of Au*			
	K	Ca	Si	N
Stander	9.9b	11.3b	7.2a	4.5c
Osiris	13.6a	12.5a	3.6a	9.4b
Zavila	9.7b	10.1c	8.0a	6.2c
UNA 80	6.3c	10.3c	7.4a	14.3a

*Means with the same letter are not significantly different according to Tukey's multiple mean test at $p < 0.05$.

TABLE-2
CHANGES IN CALCIUM, POTASSIUM, SILICON AND SODIUM LEVELS OF THE LEAF SAMPLES AS % OF GOLD USED AS COATING ON THE SURFACE OF LEAF SAMPLES TESTED FOR THE *Rhynchosporium secalis* ISOLATES INOCULATED

Genotype	Man as % of Au*			
	K	Ca	Si	N
Stander	9.9b	11.3b	7.2a	4.5c
Osiris	13.6a	12.5a	3.6a	9.4b
Zavila	9.7b	10.1c	8.0a	6.2c
UNA 80	6.3c	10.3c	7.4a	14.3a

*Means with the same letter are not significantly different according to Tukey's multiple mean test at $p < 0.05$.
†NI: Not inoculated (healthy); Iso: Scald isolate with known differential reaction [Ref. 19].

However, Na did not show such a pattern. Among the isolates studied, Iso 13 seemed to cause maximum elemental changes in the plant after inoculation. Reaction to Iso 11 was similar whereas the samples inoculated with Iso 5 had significantly different amounts of elements as compared with those inoculated by Iso 13 and Iso 11.

Gen × Iso interactions are shown in Fig. 3. Except for Zavila and UNA 80 on the left side of the graphs, all other genotypes were inoculated with the scald isolates Iso 13 (left) and Iso 11 (right). Osiris exhibited resistant reactions to the isolates, Iso 11 and Iso 13. Also, Zavila and UNA 80 on the left side of the figure were resistant to Iso 14 and Iso 18, respectively. The presence of significant interactions indicated that not only the differential responses of a given genotype to different isolate treatments could be present, but also there could be differences among genotypes in terms of the extent of changes in their levels of elemental compositions under different inoculation treatments. For example, the level of Ca in uninoculated samples of Osiris was significantly different from that of inoculated with Iso 13. There were also no differences among the isolate treatments of Osiris for the element K.

The rank and order of genotypes also changed depending on the element and inoculation treatment. The rates of these changes were also different while using

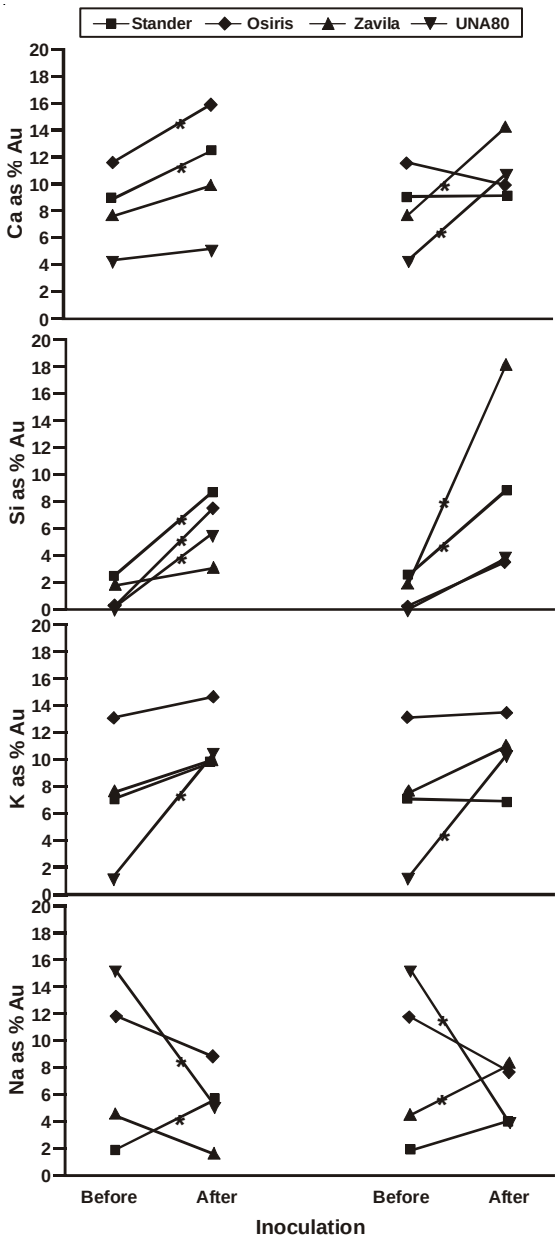

Fig. 3. Trends showing changes in calcium, silicon, potassium and sodium content of barley epidermal cells as percentage of gold used as coating on the surface of samples for scanning electron microscopy and X-ray microanalysis, before and after inoculation of barley genotypes (Stander, UNA 80, Zavila, and Osiris) with *Rhynchosporium secalis* isolates (Iso). All genotypes on the right have been inoculated with Iso 11. Stander and Osiris, UNA 80 and Zavila (on the left) have been inoculated with Iso 13, Iso 18, Iso 14, respectively. Significant elemental changes before and after inoculation are shown by asterisks

different isolates of scald. A general increasing trend was observed for Ca, Si and K in both compatible and incompatible interactions; whereas, Na, in most cases, showed decreasing trends after inoculation. The level of calcium was higher in Osiris once inoculated with Iso 13 (score 0.3 ± 0.4) than when inoculated with Iso 11 (score 1.7 ± 0.5). Calcium levels in compatible reactions of UNA 80 and Zavila showed higher increases as compared to those in incompatible interactions. Calcium levels in Stander after inoculation with Iso 11 and Iso 13 showed two different trends as calcium did not change significantly after the latter treatment but increased after the former. The increasing levels of silicon were also observed in resistant interaction. Zavila showed an increasing level of silicon upon inoculation with Iso 11 where Osiris and UNA 80 showed the least increase in their silicon levels. Potassium had its maximum level in Osiris. However, it did not differ significantly upon inoculation. Similar trends were observed for Zavila whereas UNA 80 showed significant increases in its K level after inoculation. Conversely, UNA 80, while showing the highest levels of Na, showed significant decreases after inoculation. Interestingly, a general decreasing level of Na was observed in most studied samples upon inoculation. The results indicated that changes in the levels of these elements might be inter-related.

Conclusion

SEM and XRM spectra of barley leaf surfaces inoculated with different isolates of *R. secalis* showed Ca/K-containing crystals of various shapes. It was also shown that the extent of changes in the elemental composition of epidermal cells was dependent on the genotypes and interactions with scald isolate(s). Ca and K were commonly detected at very high levels in the crystals/druses observed in slow-scalding lines, Zavila and UNA 80 similar to those observed in the susceptible cultivar Stander. For Osiris, bipyramidal crystals containing Ca/Si on the subcuticular mycelia were likely formed directly as a resistant reaction to reduce the production of stroma and to prevent subsequent sporulation. The formation of this type of crystals might also function to reduce the severity of scald in the slow-scalding lines. Similar conclusions can be drawn for the susceptible genotype Stander, which formed such druses found on the subcuticular mycelia of scald through its epidermis. Further investigations on resistance and pathogenicity mechanisms in the barley-scald pathosystem are required to interpret the results obtained.

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