Calcium Nutrition and Resistance of Alfalfa to *Ditylenchus dipsaci*[1]

ROBERT T. SHERWOOD[2] AND DONALD HUISINGH[3]

Abstract: Stem nematode-susceptible 'Atlantic' and resistant 'Lahontan' alfalfa seedlings, grown in sand and watered with complete nutrient solutions containing 0.75, 1.5, 3.0, 6.0, or 12.0 mM Ca^{++}/liter, were inoculated with *Ditylenchus dipsaci* (the stem nematode) 5–6 days after emergence. Approximately equal numbers of nematodes entered the tissues of each variety/Ca^{++} concentration within 2 days. Penetration was reduced at 12 mM Ca^{++}/liter. Reproduction during 21 days following inoculation yielded 3-fold, or greater, nematode increases in 'Atlantic' buds at all Ca^{++} concentrations, in 'Atlantic' cotyledons at the four lower concentrations, in 'Lahontan' buds at the lowest concentration and in 'Lahontan' cotyledons at the two lowest concentrations. Reproduction was lower at the higher Ca^{++} concentrations.

Increased nutrient Ca^{++} concentrations resulted in increased Ca^{++} content, decreased Na^{+} and K^{+} content, and unchanged Mg^{++} content of buds and cotyledons. Accordingly, increased nutrient Ca^{++} resulted in increased divalent/monovalent cation ratios ($Ca^{++} + Mg^{++}/Na^{+} + K^{+}$). Resistance to reproduction was correlated more closely with the divalent/monovalent cation ratio than with Ca^{++} content of tissue. At the four higher nutrient Ca^{++} concentrations, 'Lahontan' buds had higher ratios than 'Atlantic,' and infected buds had higher ratios than noninfected buds. Although cation balance modifies disease expression, the basic resistance mechanism remains unknown. *Key Words: Ditylenchus dipsaci,* Stem nematode, Alfalfa, *Medicago sativa,* Resistance, Reproduction, Calcium, Magnesium, Potassium, Sodium.

Invasion of susceptible plants by *Ditylenchus dipsaci* (Kühn) Filipjev leads to separation of host parenchyma cells. Resistant varieties show little cell separation (6, 11). Cell separation may be due to dissolution of the middle lamella by pectic enzymes. Homogenates of *D. dipsaci* contain pectic enzymes (15, 17). Muse et al. (17) detected pectic enzyme activity in infected peas. We have also found pectic enzymes in 'Atlantic' alfalfa (*Medicago sativa* L.) buds infected by *D. dipsaci* (unpublished), in contrast to an earlier negative report (14) for this crop. Seinhorst (19) postulated that dissolution of the middle lamella is essential to feeding and reproduction by the nematode.

Received for publication 19 January 1970.

[1] Supported by Cooperative Agreement No. 12-14-100-5573 (34) of the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture. Journal Series Paper No. 3067, North Carolina Agricultural Experiment Station, Raleigh, North Carolina 27607.

[2] Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Raleigh, North Carolina.

[3] Department of Plant Pathology, North Carolina State University, Raleigh. Statistical assistance by Dr. L. A. Nelson, and technical assistance by Lieselotte Conniff, Margaret Cox, Sophia Faber, Salley McCauley, Marvin Williams, and the late Bonnie Holland is gratefully acknowledged.

For several plant diseases in which pectic enzymes have been implicated, increased calcium (Ca^{++}) content of plant tissue correlates with increased resistance (1, 4, 5, 7, 20). Probably Ca^{++} forms enzyme-resistant complexes with polygalacturonates in the middle lamella (5).

The present study was undertaken to determine whether Ca^{++} nutrition influences the resistance of alfalfa to stem nematode. Initial findings were summarized elsewhere (10).

Materials and Methods

Stem nematode-susceptible 'Atlantic' and resistant 'Lahontan' alfalfa were grown in chambers providing 13 hr light at 8,000–10,000 lux, daily. Light/dark period temperatures were 24/22 C for 4 days after planting and 18/16 C thereafter. Seeds were broadcast on quartzite sand in 1-liter, bottom-drained, polyethylene containers and covered with vermiculite. The experiment was established in three chambers with each chamber serving as one replication. It was necessary to pool 30–40 seedlings from each

of three pots to provide sufficient material for one replication of a treatment.

The pots were flushed daily with modified half-strength Hoagland's solution containing $CaCl_2$ at 0.75, 1.5, 3, 6, or 12 mmole/liter. The solution also contained, per liter; 2 mmole KNO_3, 2 mmole $NaNO_3$, 1 mmole KH_2PO_4, 1 mmole $MgSO_4 \cdot 7H_2O$, 500 μg FeEDTA, 625 μg H_2BO_3, 375 μg $MnCl_2 \cdot 4H_2O$, 25 μg $ZnCl_2$, 12 μg $CuCl_2 \cdot H_2O$, and 15 μg $(NH_4)_6 MoO_4 \cdot 4H_2O$.

The seedlings were inoculated with nematodes of the Waynesville, N. C. population (2) of *D. dipsaci*. Inoculum, grown in monoxenic culture on alfalfa callus tissue, was collected, washed, and concentrated by the procedures of Krusberg (13). Inoculum was applied as one drop of a 1% carboxymethylcellulose suspension, containing about 40 active nematodes, placed on the bud of each seedling on the fifth day after planting. The pots were covered with lids. The plants were reinoculated 1 day later by covering the seedlings with additional vermiculite moistened with nutrient solution (containing Ca^{++} at the treatment concentration) and pipetting about 1200 nematodes/pot on the vermiculite. Noninoculated controls were established at each Ca^{++} concentration and received vermiculite moistened with nutrients. Pot lids and the additional vermiculite were removed 2 days later.

Nematode penetration 2 days after the second inoculation, and reproduction 20–21 days after inoculation were estimated by counting nematodes and eggs in 15 stained plants/replication. Plants were stained with hot acid fuchsin-lactophenol. Diameter of the cotyledonary node was measured under a dissecting microscope.

For chemical analyses, the seedlings were washed and divided into roots, cotyledons, and 'buds.' The 'bud' included the area which contained the apical bud and cotyledonary node at the time of inoculation; i.e., the area where swelling of susceptible seedlings occurred. The tissues were oven-dried at 100 C and ground in a Wiley mill to pass a 60-mesh screen. Duplicate 25-mg samples of plant material from each replication were wet-ashed in 3 ml HNO_3 and 1 ml perchloric acid. The residues were diluted to readable volumes with double glass-distilled water, and were analyzed by atomic absorption spectroscopy for Ca^{++} and Mg^{++} and by flame photometry for Na^{+} and K^{+}.

RESULTS

PENETRATION: Penetration of buds + cotyledons at 2 days, was approximately equal in 'Atlantic' and 'Lahontan' at each Ca^{++} concentration (Fig. 1-A, B). However, more nematodes entered cotyledons than buds of 'Lahontan,' while the opposite trend occurred in 'Atlantic.' Penetration was reduced in plants given 12 mmole Ca^{++}/liter.

REPRODUCTION: Between the second and 21st days after inoculation, the number of nematodes increased more than three-fold in 'Atlantic' buds at all Ca^{++} concentrations, in 'Atlantic' cotyledons at the four lower concentrations, in 'Lahontan' buds at the lowest concentration, and in 'Lahontan' cotyledons at the two lower concentrations (Fig. 1-A, B). The increase was less than 3-fold in 'Atlantic' cotyledons, 'Lahontan' buds, and 'Lahontan' cotyledons at the higher concentrations. 'Atlantic' buds that received 12 mmole Ca^{++}/liter had significantly fewer nematodes than at the four lower concentrations, probably because of reduced penetration (Fig. 1-A).

Since the vermiculite-inoculum covering was removed at 2 days, the increase in nematode numbers was due to reproduction. The number of eggs in buds and cotyledons at 21 days (Fig. 1-C) was correlated with the increase in nematode numbers.

SWELLING: The diameter of the cotyle-

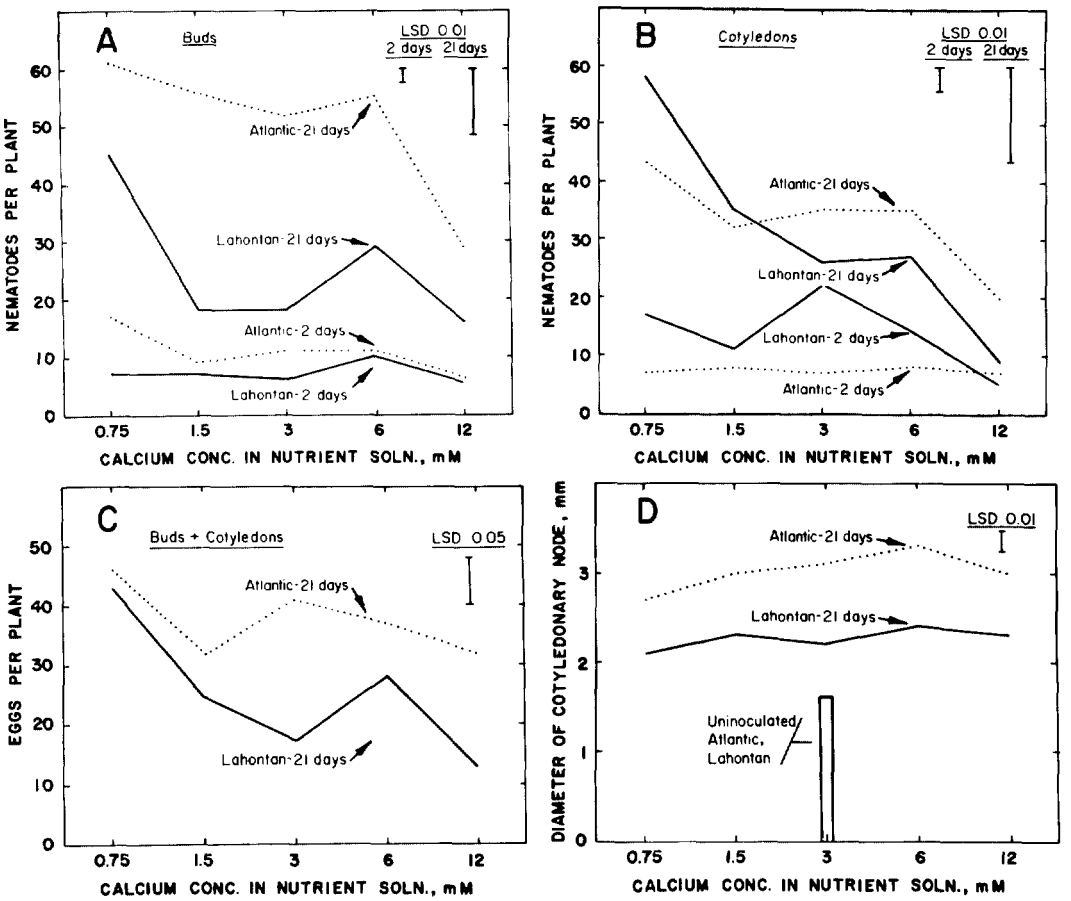
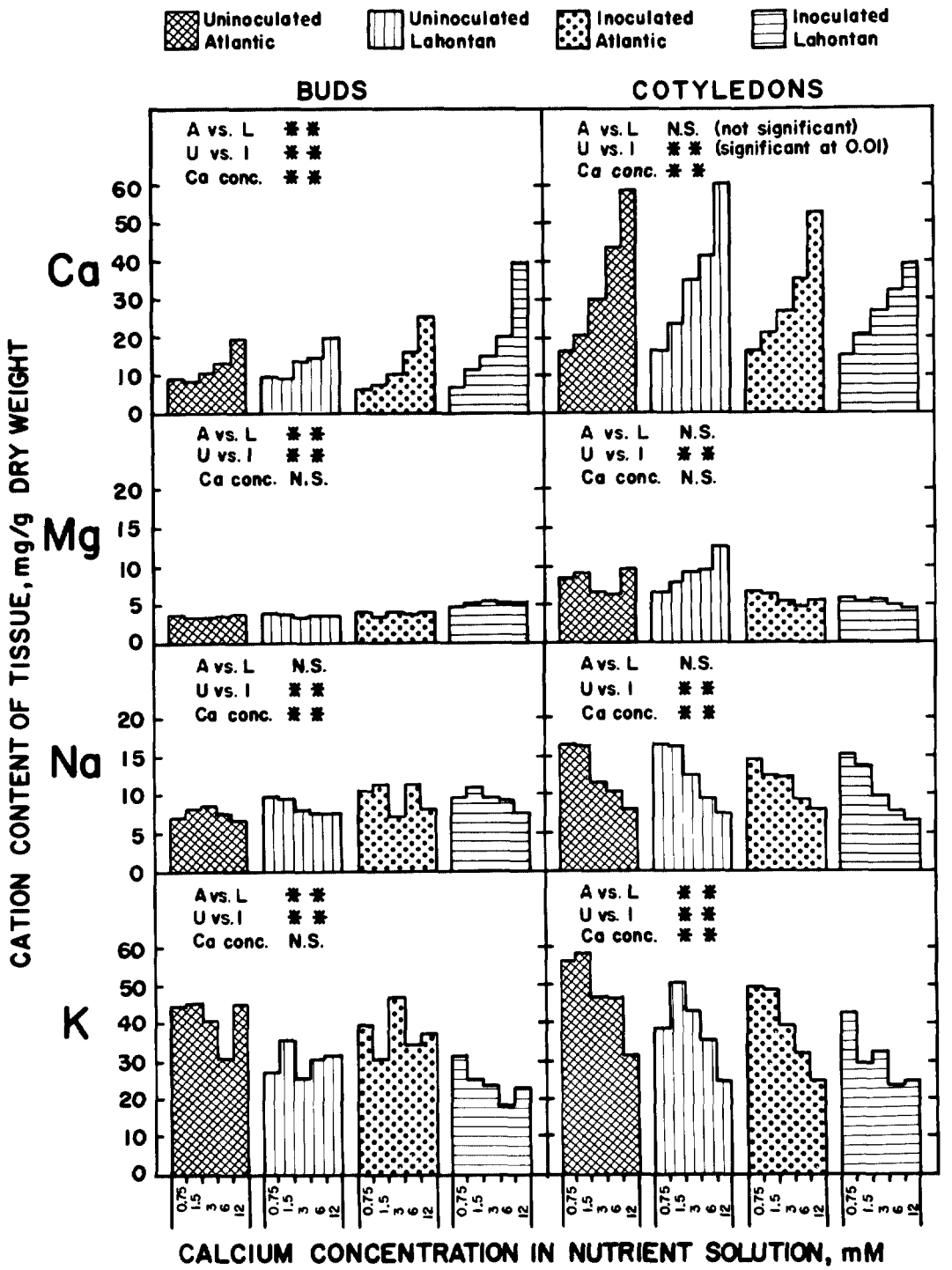


FIG. 1. Effect of nutrient solution Ca^{++} concentration on nematode numbers and disease expression in 'Atlantic' (susceptible) and 'Lahontan' (resistant) alfalfa seedlings inoculated with *Ditylenchus dipsaci*. **A, B.** Number of nematodes per seedling in (**A**) buds and (**B**) cotyledons 2 and 21 days after inoculation; **C.** Number of eggs per seedling 21 days after inoculation; **D.** Diameter of the cotyledonary node 21 days after inoculation. The open bar shows the diameter of the cotyledonary node of uninoculated 'Atlantic' and 'Lahontan' plants, of the same age, supplied 3 mM Ca^{++}/liter.

donary node of inoculated 'Atlantic' was significantly greater than that of inoculated 'Lahontan' at each Ca^{++} concentration (Fig. 1-D). Both varieties showed least swelling at the lowest Ca^{++} concentration. Diameters of noninoculated controls were not greatly affected by Ca^{++} concentration. At 21 days, noninoculated plants at the three intermediate Ca^{++} concentrations were taller and more vigorous than noninoculated plants at the

→

FIG. 2. Effect of nutrient solution Ca^{++} concentration on the Ca^{++}, Mg^{++}, Na^{+}, and K^{+} content of buds and cotyledons of uninoculated and inoculated 'Atlantic' (susceptible) and 'Lahontan' (resistant) alfalfa 21 days after inoculation with *Ditylenchus dipsaci*. Statistical significance is shown for the comparisons of mean cation contents of 'Atlantic' vs. 'Lahontan' (A vs. L), uninoculated vs. inoculated plants (U vs. I), and various Ca^{++} concentrations (Ca^{++} conc.).


Uninoculated Atlantic
Uninoculated Lahontan
Inoculated Atlantic
Inoculated Lahontan
BUDS
COTYLEDONS
A vs. L ** U vs. I ** Ca conc. **
A vs. L N.S. (not significant) U vs. I ** (significant at 0.01) Ca conc. **
A vs. L ** U vs. I ** Ca conc. N.S.
A vs. L N.S. U vs. I ** Ca conc. N.S.
A vs. L N.S. U vs. I ** Ca conc. **
A vs. L N.S. U vs. I ** Ca conc. **
A vs. L ** U vs. I ** Ca conc. N.S.
A vs. L ** U vs. I ** Ca conc. **
Ca
Mg
Na
K
CATION CONTENT OF TISSUE, mg/g DRY WEIGHT
0.75 1.5 3 6 12
CALCIUM CONCENTRATION IN NUTRIENT SOLUTION, mM

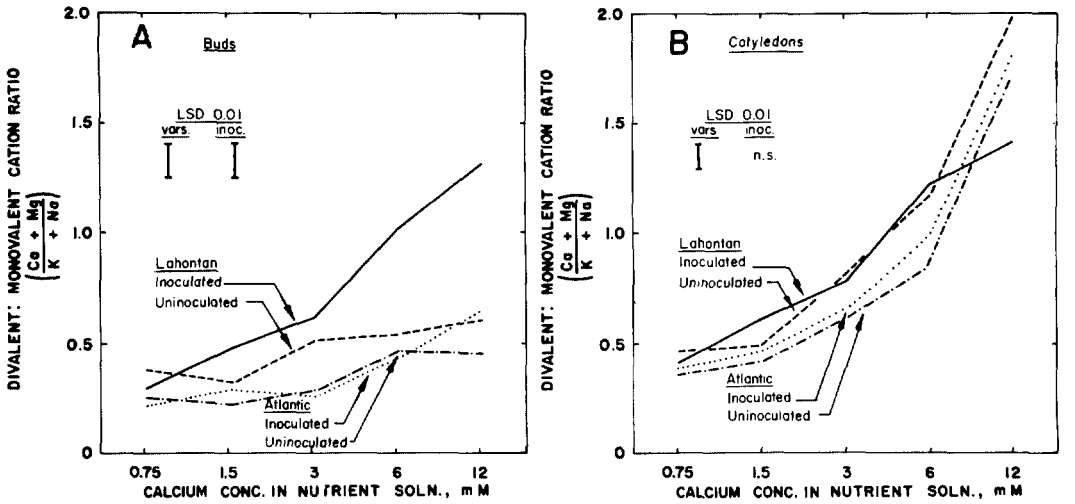


FIG. 3. Effect of nutrient solution Ca^{++} concentration on the divalent/monovalent cation ratio ($Ca^{++} + Mg^{++}/K^{+} + Na^{+}$) in (**A**) buds and (**B**) cotyledons of 'Atlantic' and 'Lahontan' alfalfa 21 days after inoculation with *Ditylenchus dipsaci*.

lowest and highest concentrations, indicating that the low and high concentrations were less suitable for host growth.

CATION CONTENT: With an increase in nutrient Ca^{++} concentration, the Ca^{++} content of buds and cotyledons increased, Na^{+} content of both parts decreased, and K^{+} content of cotyledons decreased (Fig. 2). These changes were significant ($P = 0.01$). As Ca^{++} concentration was increased, Mg^{++} content remained unchanged. The net effect was a significant increase in the divalent/monovalent cation ratio ($Ca^{++} + Mg^{++}/Na^{+} + K^{+}$) of the tissues (Fig. 3-A, B). At any given Ca^{++} concentration, cotyledons had a higher Ca^{++} content and divalent/monovalent ratio than buds.

'Lahontan' buds had higher Ca^{++} and Mg^{++} and lower K^{+} content than 'Atlantic' buds ($P = 0.01$) (Fig. 2). Inoculated buds of both varieties had higher Ca^{++} and lower K^{+} than noninoculated buds. These trends occurred only at the four highest Ca^{++} concentrations. Thus, at these concentrations, inoculated 'Lahontan' buds had a significantly higher ratio of divalent/monovalent cations than did inoculated 'Atlantic' or uninoculated control buds (Fig. 3). The mean divalent/monovalent cation ratio was 0.60 for 'Lahontan' and 0.35 for 'Atlantic.'

In contrast to buds, inoculated cotyledons had lower Ca^{++} content than noninoculated cotyledons, and Ca^{++} content of cotyledons did not differ significantly between varieties (Fig. 2). Mg^{++}, K^{+}, and Na^{+} were lower in inoculated than noninoculated cotyledons. The divalent/monovalent cation ratio was higher in 'Lahontan' than in 'Atlantic' cotyledons (Fig. 3).

NEMATODES AND CATIONS IN ROOTS: Roots contained less than 1.5 nematodes/seedling 2 days after inoculation. There was no increase in nematode numbers up to 21 days. There were no differences in nematode numbers between varieties or between Ca^{++} concentrations.

Increases in nutrient Ca^{++} concentration increased Ca^{++}, decreased Na^{+}, and did not affect K^{+} or Mg^{++} content of roots. The Ca^{++} content and the divalent/monovalent cation ratio of roots was lower than that of buds or cotyledons at each Ca^{++} concentration.

FIELD GROWN PLANTS: Analyses were made of terminal buds from two resistant varieties and three susceptible varieties grown in replicated, normally limed, field plots showing slight infection by stem nematode. Ca^{++} content of buds was 8.8 to 11.4 μg/mg and the divalent cation ratio was 0.46 to 0.57. The differences were not highly significant.

DISCUSSION

In agreement with previous reports (8, 21), resistance of 'Lahontan' to *D. dipsaci* was expressed as reduced swelling and reproduction but not as reduced penetration. Due to genetic heterogeneity, a few 'Lahontan' plants were highly susceptible and supported most of the reproduction observed (21).

We measured Ca^{++}, Mg^{++}, Na^{+}, and K^{+} content of tissues because: (i) earlier reports (12, 18) indicated that Ca^{++} supply influences uptake of other cations as well as Ca^{++}; (ii) these were the only cations supplied in appreciable amounts; and (iii) it seemed logical that crosslinkage of pectic substances by divalent cations would be influenced more by the ratio of divalent/monovalent cations than by the concentration of any one cation. The latter consideration led us to include Mg^{++} in calculating the divalent/monovalent cation ratio, even though Mg^{++} content of tissues was not influenced by Ca^{++} concentration of the nutrient solution. The decrease in Na^{+} and K^{+} content of tissue brought about by an increase in nutrient Ca^{++} concentration was consistent with cation absorption patterns in intact barley roots (12, 18).

Increased Ca^{++} content and increased divalent/monovalent cation ratio of buds and cotyledons were accompanied by decreased nematode reproduction. Ca^{++} content of tissues was not an absolute determinant of resistance. For example, reproduction was less than 3-fold in 'Lahontan' buds containing 12 mg Ca^{++}/g, but greater than 4-fold in 'Atlantic' cotyledons which contained 35 mg Ca^{++}/g. The divalent/monovalent cation ratio was more closely related to resistance than was Ca^{++} content. With ratios less than 0.5, reproduction was high (3- to 6-fold), and with ratios greater than 0.8 reproduction was lower (less than 3-fold).

'Lahontan' buds exposed to the four higher Ca^{++} concentrations were resistant, but 'Atlantic' were not. This pattern was correlated with a higher divalent/monovalent cation ratio in inoculated 'Lahontan' than in 'Atlantic' at these concentrations. Thus, differences in resistance of varieties might be related to differences in their ability to accumulate cations.

Inoculated 'Lahontan' buds had higher Ca^{++} and Mg^{++} content than noninoculated buds. This suggests that 'Lahontan' buds may possess a divalent cation accumulation mechanism which is stimulated by infection. This might be similar to the accumulation mechanism described by Bateman and coworkers (3, 4, 5) to account for induceable resistance of beans to *Rhizoctonia solani* Kühn. It is possible that increases in divalent/monovalent cation ratios during infection proceeded from alterations which were not related to a resistance mechanism. For example, intensified breakdown of storage or structural compounds in infected tissues could give apparent increases in the immobile divalent cations.

The size of an infected bud was not always related to the number of nematodes it contained. For example, buds grown with the lowest nutrient Ca^{++} concentration showed least enlargement but supported the greatest number of nematodes. Swelling results from cell multiplication and from enlargement of separated cells (13). Perhaps, cell separation [considered by Seinhorst (19) to be essential for nematode feeding and reproduction] was greatest at the low Ca^{++} concentration; while cell enlargement or multiplication [considered by Seinhorst (19) not to be essential for re-

production] was greatest at Ca^{++} concentrations which were most favorable for host growth, i.e., the intermediate concentrations.

The effect of Ca^{++} upon reproduction was compatible with the theory that resistance may be modified by crosslinking the pectic substances of middle lamellae with divalent cations. However, there are other mechanisms by which cations might modify resistance. Plants supplied the high Ca^{++} concentration were brittle. Perhaps brittle or rigid tissues are less readily penetrated by nematodes, or are ruptured by nematode movements and rendered unsuitable for feeding. In this connection, Barker and Sasser (2) observed disruption and necrosis of 'Lahontan' tissues. Alternatively, cations may exert a direct effect upon nematode metabolism rather than upon host tissues.

Resistance of 'Lahontan' buds and cotyledons broke down at the low Ca^{++} concentration. This might have been due to a weakened middle lamella; or it might have involved other effects of Ca^{++} deficiency such as altered cell membrane structure (16) and permeability (9).

The effect of Ca^{++} concentration upon nematode reproduction in 'Atlantic' was similar to that previously noted in the susceptible variety 'DuPuits' (10). The effect upon reproduction in 'Lahontan' corresponded with that noted in the resistant variety 'Nl'. The present tests, unlike the earlier ones, showed differences in Ca^{++} content between noninoculated and inoculated plants and between varieties. Much less infection was obtained in the previous tests with 'DuPuits' and 'Nl', because inoculum was applied only once. Thus differential accumulation of cations was not induced in the previous tests.

Our studies indicate that the divalent/monovalent cation content of tissue modifies the expression of resistance by alfalfa buds. The basic nature of resistance remains unknown. Certainly, cation modification is not involved in the failure of *D. dipsaci* to reproduce in roots. Nor would it account for restrictions in host ranges of the biological races of *D. dipsaci*. The cation contents of field grown plants were similar to those which permitted the expression of susceptibility by 'Atlantic' and resistance by 'Lahontan' in the laboratory.

Literature Cited

1. Alghisi, P., and G. Ferraza. 1967. Influenza esercitata dal sulla natura della sostanze pectiche presenti in piante di Radicchio e sulla resistenza di queste alla *Sclerotinia minor*. Riv. Patol. Veg. Pavia Ser. 4, 3: 145–160.
2. Barker, K. R., and J. N. Sasser. 1959. Biology and control of the stem nematode, *Ditylenchus dipsaci*. Phytopathology 49: 664–670.
3. Bateman, D. F. 1964. An induced mechanism of tissue resistance to polygalacturonase in *Rhizoctonia*-infected hypocotyls of bean. Phytopathology 54:438–445.
4. Bateman, D. F., and R. D. Lumsden. 1965. Relation of calcium content and nature of the pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of *Rhizoctonia solani*. Phytopathology 55: 734–738.
5. Bateman, D. F., and R. L. Millar. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4:119–146.
6. Blake, C. D. 1962. The etiology of tulip-root disease in susceptible and resistant varieties of oats infested by the stem nematode, *Ditylenchus dipsaci* (Kühn) Filipjev. II. Histopathology of tulip-root and development of the nematode. Ann. Appl. Biol. 50: 713–722.
7. Corden, M. E. 1965. Influence of calcium nutrition on *Fusarium* wilt of tomato and polygalacturonase activity. Phytopathology 55:222–224.
8. Griffin, G. D. 1968. The pathogenicity of *Ditylenchus dipsaci* to alfalfa and the relationship of temperature to plant infection and susceptibility. Phytopathology 58:929–932.
9. Hanson, J. 1965. Metabolic aspects of ion transport. p. 63–74. *In* F. Greer and T. Armey (ed.) Genes to genus; a symposium on plant growth. International Mineral and Chemical Corp., Skokie, Ill.
10. Huisingh, D., and R. T. Sherwood. 1968. The role of calcium in resistance of alfalfa to *Ditylenchus dipsaci*. Nematological 14: 8–9. (Abstr.)

11. HUSSEY, R. S., AND L. R. KRUSBERG. 1968. Histopathology of resistant reactions in Alaska pea seedlings to two populations of *Ditylenchus dipsaci*. Phytopathology 58: 1305–1310.
12. JOHANSEN, C., D. G. EDWARDS, AND J. F. LONERAGEN. 1968. Interactions between potassium and calcium in their absorption by intact barley plants. II. Effects of calcium and potassium concentration on potassium absorption. Plant Physiol. 43:1722–1726.
13. KRUSBERG, L. R. 1961. Studies on the culturing and parasitism of plant-parasitic nematodes, in particular *Ditylenchus dipsaci* and *Aphelenchoides ritzemabosi* on alfalfa tissues. Nematologica 6:181–200.
14. KRUSBERG, L. R. 1963. Effect of galling by *Ditylenchus dipsaci* on pectins in alfalfa. Nematologica 9:341–346.
15. KRUSBERG, L. R. 1967. Pectinases in *Ditylenchus dipsaci*. Nematologica 13:443–451.
16. MARINOS, N. G. 1962. Studies on submicroscopic aspects of mineral deficiencies. I. Calcium deficiency in the shoot apex of barley. Amer. J. Botany 49:834–841.
17. MUSE, BARBARA D., L. D. MOORE, R. R. MUSE, AND A. S. WILLIAMS. 1970. Pectolytic and cellulolytic enzymes of two populations of *Ditylenchus dipsaci* on 'Wando' pea (*Pisum sativum* L.). J. Nematol. 2:118–124.
18. RAINS, D. W., AND E. EPSTEIN. 1967. Sodium absorption by barley roots: its mediation by mechanism 2 of alkali cation transport. Plant Physiol. 42:319–323.
19. SEINHORST, J. W. 1959. The genus *Ditylenchus* and related genera. p. 33–43. *In* J. F. Southey (ed.) Plant Nematology. Great Britain Ministry of Agr., Tech Bull. 7, 175 pp. Her Majesty's Stationery Office, London.
20. THOMAS, C. A. 1966. Calcium and water-insoluble pectic substances in safflower hypocotyls in relation to resistance to *Phytophthora drechsleri*. Phytopathology 56:985–986.
21. WYNN, J. D., AND T. H. BUSBICE. 1968. Effects of temperature and incubation period on the expression of resistance to stem nematode in alfalfa. Crop Sci. 8:179–183.