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Osmolyte Allocation in Response to *Tylenchulus semipenetrans* Infection, Stem Girdling, and Root Pruning in Citrus[1]

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Abstract: Previous studies indicated that *Tylenchulus semipenetrans* infection reduced concentrations of inorganic osmolytes, (Na^+, Cl^-, K^+), in roots, along with leaf K^+ in citrus. However, infection increased leaf Na^+ and Cl^-, along with carbohydrates in roots. Pruning of roots also increased carbohydrates in intact roots, whereas shoot pruning increased carbohydrates in shoots. Carbohydrates are translocated as reducing sugars, which collectively form organic osmolytes. Because changes in concentrations of osmolytes regulate osmotic potential in plant cells, we hypothesize that increasing concentrations of organic osmolytes in an organ displaces inorganic osmolytes. We measured the osmotic potentials of young citrus trees under nematode infection, stem girdling, and root pruning at two salinity levels. All treatments reduced leaf osmotic potentials at four sampling times. At harvest, 16 days after pruning and girdling treatments, organs with higher carbohydrates had lower inorganic osmolytes and vice versa, regardless of the treatment. Pruning simulated effects of nematode infection, whereas girdling reduced the effects of nematodes. Results suggested that high organic osmolytes in roots displace inorganic osmolytes, thereby avoiding very low osmotic potentials.

Key words: carbohydrates, chloride, citrus nematode, osmolyte, potassium, root pruning, sodium, stem girdling, *Tylenchulus semipenetrans*.

After *Tylenchulus semipenetrans* reproduction and death, the cortical layer of citrus roots ''sloughs'' off, resulting in a root pruning-like effect that reduces the root/shoot ratio (O'Bannon and Esser, 1985). Because this nematode is a non-aggressive feeder, its deleterious effects on citrus root growth occurs through this ''sloughing'' off. The re-growth of roots re-establishes the pre-''sloughing'' root/shoot ratio and determines the magnitude and direction toward which photosynthates are mobilized. In actively growing sour orange (*Citrus aurantium* L.) seedlings, the normal pre-pruned root/shoot ratio was re-established within 14 days (Mashela, 1992), suggesting that non-cumulative responses (like photosynthesis and transpiration) may no longer respond to the pruning. Cumulative responses, such as the accumulation of non-structural carbohydrates in the form of starch and the accumulation of ions, however, may remain for some time in plant tissues after the normal root/shoot ratio has been re-established.

A split-root pruning technique was developed to ensure that ion fluxing problems that can be encountered when using traditional root-pruning techniques in salinity studies were avoided (Mashela, 1992). The technique precluded contact of the severed portions with soil solutions, thus preventing ion fluxes through pruning wounds into soil solutions. The technique allowed for the modification of salinity soon after pruning, without waiting for pruning wounds to heal, missing the point at which organic osmolytes are still in excess to the requirements of maintenance and growth. Also, the technique facilitated data interpretation by preventing pruned plants from re-establishing pre-pruned root/shoot ratios prior to initiating salinity. Compartmentation also eliminated the entangling of roots, thereby avoiding damage to the remaining roots during removal of the excised halves.

Photosynthates, translocated as sucrose, form a large portion of organic osmolytes, whereas inorganic osmolytes comprised Na^+, Cl^-, and K^+ ions (Salibury and Ross, 1985; Waisel, 1972). Osmolytes modify osmotic potentials in plant cells, thereby avoiding damaging osmotic potentials (Waisel, 1972). Effects of *T. semipenetrans* infection on inorganic osmolytes were not conclusive in various studies (Fouche et al., 1977; Labanauskas et al., 1965; Milne and Willers, 1979; Van Gundy and Martin, 1961). Measurable responses on inorganic osmolytes were induced consistently when high densities of *T. semipenetrans* per plant were used for inoculation (Mashela, 1992; Mashela and Nthangeni, 2002). Generally, high densities of *T. semipenetrans* increased Na^+ and Cl^- in leaves and decreased the two ions in roots, but decreased K^+ in both leaves and roots. Tarjan and O'Bannon (1984) proposed that *T. semipenetrans* infection changed the partitioning of Na^+ and K^+ in citrus either by physical damage to root cells or through changes in the permeability of cells, causing roots to uptake greater concentrations of Na^+ and less of K^+ ions.

We hypothesize that increasing organic osmolytes in tissues regulates osmotic adjustments in cells through the storage of organic osmolytes in non-osmotic forms and by mobilizing inorganic osmolytes to organs with lower concentrations of organic osmolytes, thereby inducing the upset in the partitioning of Na^+, K^+, and Cl^- ions. Stem girdling and root pruning may offer some explanation on how *T. semipenetrans* infection upsets the partitioning of osmolytes. The objective of this study was to evaluate the responses of osmotic potential, concentrations of inorganic osmolytes, and non-

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structural carbohydrates under *T. semipenetrans* infection, mechanical root pruning, and stem girdling in short-term experiments in the presence and absence of salinity stress.

Materials and Methods

The study was conducted in the greenhouse at the Horticultural Unit of the University of the North, South Africa (23°53′10″S, 29°44′15″E) on 18 October 1999. Thirteen-month-old Palmer navels on rough lemon (*Citrus jambhiri* Hush) rootstocks were obtained from Du Roi nursery (Box 66, Letsitele, RSA). Taproots were vertically split in two from the apex to 15 cm above the soil surface, and the joint was firmly secured with Parafilm M (American National Can, Greenwich, CT). Roots were trimmed so that each half-root system had approximately the same number of lateral roots. Each plant was transplanted so that each of the two halves occupied a 30-cm-diam. plastic pot containing 2,800 cm^3 steam-pasteurized Hutton soil, consisting of 64% sand, 4% silt, 32% clay, and 1.6% organic matter. The electrical conductivity (EC) of the soil paste was 1.65 mS/m and the pH was 6.63 (Rhue and Kidder, 1983). Plants were allowed to recover and re-establish from the root systems being split and trimmed for a 5-week period prior to experimental treatments. Each pot was irrigated with 3 liters tap water having EC 2.60 mS/m every fourth day, and each half-root system was fertilized weekly using 1.5 g 2:1:2 (43) that provided a total of 0.35 mg N, 0.32 mg K, and 0.32 mg P/ml. Also, the mixture provided 0.9 mg Mg, 0.75 mg Fe, 0.075 mg Cu, 0.35 mg Zn, 1.0 mg B, 3.0 mg Mn, and 0.07 mg Mo per plant. Day and night ambient temperatures averaged 28 °C and 15°C, respectively, with maximum temperatures controlled using thermostatically activated fans. The experiment involved eight treatments: nematode infection, stem girdling, root pruning, and untreated control—each at two levels of salinity—laid out in a randomized complete block design with 10 replicates.

Nematode inocula were collected in Zebediela Citrus Estate (24°28S, 29°18E), extracted from roots in 1% NaOCl (Hussey and Barker, 1973), and incubated for 3 days using the modified Baermann method (Rodríguez-Kábana and Pope, 1981) to obtain second-stage juveniles for inoculation. Plants were inoculated with a total of 893,510 juveniles + males into each half-root system in 10-cm-deep holes on the cardinal quadrants of the trunk. Pruned, girdled, and control plants were inoculated with inoculum filtrate (25-µm-pore sieve) to establish in their rhizosphere any microbes associated with *T. semipenetrans*.

Girdling and pruning treatments were introduced 10 weeks after nematode inoculation. Girdling the stem was initiated by removing the bark and phloem in a 10-cm band at 15 cm above the graft union. Root pruning was initiated by severing half of the root system below the point at which the two halves were attached. Salinity stress was achieved by irrigating with solutions with 2 M NaCl + 0.50 M $CaCl_2$ on 0, 4, 8, 12, and 16 days after initiating pruning and girdling. Calcium chloride was included as a source of Ca^{++}, which is essential for the maintenance of cell membranes, particularly under saline conditions (Bohn et al., 1985).

One mature leaf/plant was sampled at random before dawn between 06h00 and 06h15 on irrigation days to measure osmotic potential (π). Five 1-cm-diam. discs/leaf were sealed in a scintillation vial (Fisher) and stored at −80 °C until osmotic potential was measured. Frozen leaf discs still in sealed vials were thawed at 5 °C for 12 hours. Tissue sap was pipeted onto a paper disc and its osmotic potential measured using a Wescor vapor-pressure osmometer that was calibrated using 0.01, 0.05, 0.10, 0.50, and 1.00 molal NaCl solutions.

Sixteen days after pruning and girdling treatments began, the root systems were removed and gently immersed in water to free soil particles. Nematodes were extracted from 10 g roots/plant by maceration in a blender for 30 seconds in 1% NaOCl and then passed through a nested series of 1,000, 150, and 45-µm sieves onto a 25-µm-pore sieve (Hussey and Barker, 1973). Eggs and juveniles from the 25-µm-pore sieve were incubated for 4 days in the modified Baermann method (Rodríguez-Kábana and Pope, 1981), and juveniles were counted under the light microscope. Shoots and roots were dried for 4 days at 60 °C for dry matter analyses. Mature leaves and roots were ground separately in a Wiley mill, passed through 1-mm sieve, and Na^+ and K^+ quantified (Rhue and Kidder, 1983). A Haake chlorometer (Haake Buchler Instruments, Saddle Brook, NJ) was used to measure Cl^- concentration of leaves and roots. Glucose oxidase (Sigma) was used to analyze free glucose in the supernatant (Nelson, 1944). Soluble starch in the supernatant and insoluble starch in the pellet were analyzed with glucose oxidase following 48 hours of amyloglucosidase (Sigma, St. Louis, MO) digestion (Smith, 1981). Arsenomolybdate (Sigma) was used to analyze reducing sugars (Roe et al., 1949) and resorcinol reagent (Smith, 1981) to analyze ketone sugars. The entire study was repeated 12 January 2000 under similar conditions except that inoculation consisted of 537, 120 juveniles + males/half-root system.

Because data trends were similar in both experiments ($n = 10$), the data were pooled across the two experiments ($n = 20$). Data were subjected to analysis of variance with SAS software (SAS Institute, Inc., Cary, NC), and mean treatment differences were separated using Duncan's multiple-range test. Because osmotic potential data under low salinity and non-salinity were not different ($P \geq 0.10$), data were pooled across salinity levels ($n = 40$). Nematode data were transformed with ln $(x + 1)$ before analysis, but untransformed data are reported. Only data where the *F*-test was significant ($P \leq 0.01$) are discussed, unless otherwise indicated.

RESULTS

Nematode infection, girdling, and pruning each consistently decreased osmotic potential in leaves (Table 1). Girdling resulted in the greatest decrease in osmotic potential, suggesting increased accumulation of excess photosynthates in shoots. At the initiation of girdling and pruning, nematode infection had already reduced osmotic potential.

Root pruning increased non-structural carbohydrates in the remaining roots under both non-salinity and salinity, whereas stem girdling reduced carbohydrates in roots (Table 2). *Tylenchulus semipenetrans* infection increased root starch under both salinity and non-salinity and decreased reducing sugars under salinity. There were no treatment differences in ketone sugars. Girdling increased non-structural carbohydrates in leaves under both salinity and non-salinity; pruning increased ($P \leq 0.05$) ketone sugars only under non-salinity (Table 3).

Girdling enhanced the accumulation of inorganic osmolytes in roots under both levels of salinity, with the exception of Na$^+$ under salinity (Table 4). However, nematode and pruning treatments consistently reduced concentrations of inorganic osmolytes in roots under both levels of salinity. In leaves, girdling reduced K$^+$ but had no effect ($P \geq 0.10$) on Na$^+$ and Cl$^-$ under either salinity level except for increasing the low Na$^+$ under non-salinity (Table 5). Conversely, nematode and pruning treatments reduced K$^+$ in leaves and increased Na$^+$ and Cl$^+$.

Girdling, nematode, and pruning treatments reduced root/shoot ratio under non-salinity ($P \leq 0.05$) but had no effect ($P \geq 0.10$) under salinity (Table 6). When the root/shoot ratios under non-salinity and salinity were compared, they were not different ($P \geq 0.1$) except for that under nematode treatment.

Soil paste EC averaged 3.85 mS/m and 13.49 mS/m under non-salinity and low salinity, respectively, whereas soil pH averaged 6.93 and 5.28, respectively. Salinity level had no effect on nematode densities ($P \geq 0.10$), with the pooled average density of 9,809 juveniles + males/g fresh roots (range 703–16, 754).

TABLE 1. Osmotic potential in leaves of Palmer navel on rough lemon rootstock in the presence of *Tylenchulus semipenetrans* infection, mechanical root pruning, and stem girdling.

	Days after initiating pruning and girdling				
Treatment	0	4	8	12	16
Control	−0.18 a	−0.17 a	−0.20 a	−0.20 a	−0.19 a
Nematode	−0.35 b	−0.27 b	−0.36 b	−0.32 b	−0.35 b
Pruned	−0.18 a	−0.45 c	−0.68 c	−0.53 c	−0.30 b
Girdled	−0.19 a	−0.59 d	−0.83 d	−0.89 d	−0.93 c

Data are means (MPa) of 40 replicates pooled over two experiments and two salinity levels. Means within a column followed by a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

TABLE 2. Non-structural root carbohydrate of Palmer navel on rough lemon rootstock under *Tylenchulus semipenetrans* infection, mechanical root pruning, and stem girdling using two levels of salinity at day 16 after initiating pruning and girdling.

	Starch		Ketone sugars[a]		Reducing sugars[b]	
Treatment	No salt	Salt	No salt	Salt	No salt	Salt
Control	1.83 c	2.29 c	2.50 b	2.48 b	0.66 b	0.72 b
Nematode	2.54 b	2.92 b	2.64 b	2.54 b	0.52 b	0.57 c
Pruned	3.97 a	4.61 a	3.39 a	3.49 a	0.83 a	0.95 a
Girdled	1.05 d	1.57 d	1.79 c	1.61 c	0.29 c	0.32 c

Data are means (% dry weight) of 20 replicates pooled over two experiments. Means within a column followed by a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

[a] Ketone sugars = sucrose + fructose + fructans.

[b] Reducing sugars = fructose + glucose + others.

DISCUSSION

Many glycophytes are able to adjust their cellular osmotic potential to the fluctuating external osmotic potential in soil solution by changing the concentrations of inorganic osmolytes in root vacuoles (Waisel, 1972). The higher organic osmolytes and lower inorganic osmolytes in citrus roots suggest a greater importance on photosynthetic sucrose for adjusting water potentials in citrus roots subjected to salinity stress. This mechanism is well documented in glycophytes, which are inherently sensitive to salinity (Waisel, 1972). In this study we used pruning and girdling treatments to assist in explaining the mechanisms through which *T. semipenetrans* infection alters the partitioning of inorganic osmolytes in citrus.

Pruning and girdling data suggested that *T. semipenetrans* infection did not alter the partitioning of Na$^+$ and K$^+$ exclusively through physical damage to root cells as previously postulated (O'Bannon and Esser, 1985; Tarjan and O'Bannon, 1984). Girdling had similar effects on K$^+$ as did shoot pruning, where K$^+$ was redistributed from shoots to roots, resulting in high K$^+$ in roots and

TABLE 3. Non-structural carbohydrate in leaves of Palmer navel on rough lemon rootstock under *Tylenchulus semipenetrans* infection, mechanical root pruning, and stem girdling using two levels of salinity at day 16 after initiating pruning and girdling.

	Starch		Ketone sugars[a]		Reducing sugars[b]	
Treatment	No salt	Salt	No salt	Salt	No salt	Salt
Control	1.33 b	0.98 b	2.15 c	2.25 bc	0.87 b	0.89 b
Nematode	1.28 b	1.11 b	2.36 c	1.94 c	0.39 c	0.51 c
Pruned	1.36 b	1.07 b	3.60 b	2.38 b	0.61 bc	0.83 bc
Girdled	3.39 a	2.93 a	4.98 a	5.16 a	2.83 a	2.09 a

Data are means (% dry weight) of 20 replicates pooled over two experiments. Means within a column followed by a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

[a] Ketone sugars = sucrose + fructose + fructans.

[b] Reducing sugars = fructose + glucose + others.

TABLE 4. Concentration of potassium, chloride, and sodium ions in roots of Palmer navel on rough lemon rootstock under *Tylenchulus semipenetrans* infection, mechanical root pruning, and stem girdling using two levels of salinity at day 16 after initiating pruning and girdling.

	Potassium		Chloride		Sodium	
Treatment	No salt	Salt	No salt	Salt	No salt	Salt
Control	1.36 b	1.01 b	0.81 b	0.94 b	0.43 b	0.79 a
Nematode	0.93 c	0.96 bc	0.65 c	0.54 c	0.14 c	0.27 b
Pruned	0.89 c	0.69 c	0.19 d	0.52 c	0.08 d	0.29 b
Girdled	1.66 a	1.38 a	1.03 a	1.11 a	0.92 a	0.83 a

Data are means (% dry weight) of 20 replicates pooled over two experiments. Means within a column followed by a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

TABLE 6. Shoot and root dry weights of Palmer navel on rough lemon rootstock under *Tylenchulus semipenetrans* infection, mechanical root pruning, and stem girdling under two levels of salinity at day 16 after initiating pruning and girdling.

	Shoot weight (g)		Root weight (g)		Root/shoot ratio	
Treatment	No salt	Salt	No salt	Salt	No salt	Salt
Control	6.05 a	5.93 a	2.86 a	2.03 a	0.47 a	0.39 a
Nematode	5.99 a	5.48 b	1.91 b	1.84 c	0.21 c	0.34 a
Pruned	6.01 a	5.47 b	1.18 c	1.93 bc	0.38 b	0.35 a
Girdled	6.63 a	5.88 a	2.14 b	1.99 b	0.32 bc	0.34 a

Data are means of 20 replicates pooled over two experiments. Means within a column followed by a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

low K^+ in leaves (Swietlik, 1986). Girdling in this study and shoot pruning in another study (Swietlik, 1986) increased K^+, Na^+, and Cl^- in roots but had no effect on Na^+ and Cl^- in leaves. However, when roots were challenged with salinity, pruning, allelopathy, or nematode stress, K^+ reductions in roots and leaves were concurrent, suggesting that a mechanism for the redistribution of K^+ is different to that of Na^+ (Graham and Syvertsen, 1989; Mashela, 2001; Mashela, 1992; Mashela and Nthangeni, 2002; Geisler and Ferree, 1984; Waisel, 1972).

When the experiments were terminated 16 days after root pruning, pre-pruning root/shoot ratio had been re-established under salinity stress where high leaf Na^+ and Cl^- arrested shoot growth, whereas high concentrations of photosynthates in roots hastened the re-establishment of the pre-pruning root/shoot ratio (Mashela, 1992). The immediate effect of root pruning was a reduced root/shoot ratio. New growth was redistributed in favor of root growth, with high concentration of photosynthates being diverted toward this effort (Mashela, 1992). The lower osmotic potential in leaves soon after initiating pruning and girdling suggested that the plants in these treatments were producing excess quantities of photosynthates. In actively growing sinks, sucrose is hydrolyzed into glucose and fructose that are used in metabolism, and excesses can be stored in non-osmotic forms such as starch and in less osmotic forms such as ketone sugars (Salisbury and Ross, 1985). The accumulation of starch in roots of pruned and nematode treatments in this and another study (Mashela, 1992) suggested that the rate of sucrose delivery to roots exceeded that of metabolism.

TABLE 5. Concentration of potassium, chloride, and sodium ions in leaves of Palmer navel on rough lemon rootstock under *Tylenchulus semipenetrans* infection, mechanical root pruning, and stem girdling using two levels of salinity at day 16 after initiating pruning and girdling.

	Potassium		Chloride		Sodium	
Treatment	No salt	Salt	No salt	Salt	No salt	Salt
Control	1.64 a	0.95 a	0.06 c	0.13 c	0.04 d	0.39 c
Nematode	0.84 b	0.73 b	0.15 b	0.73 b	0.21 b	0.57 b
Pruned	0.66 c	0.70 b	0.21 a	1.03 a	0.38 a	0.90 a
Girdled	0.63 c	0.27 c	0.04 c	0.11 c	0.13 c	0.32 c

Data are means (% dry weight) of 20 replicates pooled over two experiments. Means within a column followed by a common letter are not different according to Duncan's multiple-range test ($P \leq 0.05$).

Generally, the concentrations of ions in root symplasm are several thousands-fold higher than the concentrations in soil solutions, whereas those in apoplasm are similar to those in soil solutions (Bohn et al., 1985; Waisel, 1972). Under NaCl salinity, Na^+ and Cl^- in soil solution are high; thus, the two ions have competitive advantages over other ions on the absorption sites of roots (Bohn et al., 1985). Under salinity stress, root cells of glycophytes accumulate large quantities of carbohydrates (Waisel, 1972). Thus, Na^+ and Cl^- cannot accumulate in vacuoles of root cells because of high organic osmolytes in these cells. The route of minimal resistance for the two ions is into the xylem vessels, where the ions are passively transported to leaves through the transpiration stream. The two ions accumulate in leaves, with leaf abscission serving as the principal route through which glycophytes rid themselves of excess Cl^- and Na^+ (Waisel, 1972). However, because levels of K^+ in soil solution are low, when K^+ is excreted into apoplasm the ion leaches out into soil solution, where it becomes relatively unavailable compared to the more concentrated and competitive Na^+ ions at the absorption sites (Bohn et al., 1985; Salisbury and Ross, 1985).

Potassium is required for activating starch synthase that hydrolyzes sucrose into glucose and fructose (Salisbury and Ross, 1985). If K^+ becomes deficient in roots, foliar K^+ is mobilized to the roots where its role in catalyzing starch synthase prevents excess accumulation of sucrose, which would otherwise counteract the role of ion excretion into the apoplast during adjustments of water potential. However, as more sucrose arrives in roots, K^+ from leaves is further excreted into the root apoplast, where it leaches into the soil, following the route of minimal resistance, resulting in both leaf and root K^+ deficits. Evidence suggested that when plants were subjected to stresses that increased carbohydrate concentrations in roots, cells could not absorb K^+ that was in the apoplast. Fouche et al. (1977) demonstrated that increasing soil K^+ without reducing nematode densities did not improve K^+ levels in leaves. Also, when K^+ levels in soil solution were augmented through fertilizers, K^+ followed the same route as Cl^- and Na^+, provided the normal citrus root/shoot ratio had been re-established (Graham and Syvertsen, 1989). However, when the root/shoot ratio was not re-established, the continual increase of carbohydrates in roots prevented the accumulation of K^+ in root cells.

Based on pruning and girdling data, the mechanism whereby *T. semipenetrans* reduces inorganic osmolytes in roots appears to be a four-step process: (i) Nematode parasitism reduces the root/shoot ratio through the ''sloughing'' off effect of rootlets, which has a root-pruning effect. (ii) The plant then diverts more organic osmolytes to roots in response to the lowered root/shoot ratio. (iii) In roots, organic osmolytes decrease water potential. (iv) As a measure to avoid very low osmotic potentials in root cells, excess organic osmolytes are stored in non-osmotic forms such as starch, whereas inorganic osmolytes are displaced from the vacuoles of root cells.

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