Variability of Nitrate Levels in Sweetclover Seedlings†

Ken A. Bassendowski and Alister D. Muir*

Agriculture Canada Research Centre, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada

Nitrate concentrations in cotyledons of 8–10-day-old field-grown seedlings of yellow sweetclover (*Melilotus officinalis* cv. Norgold) were determined by HPLC ion chromatography over a 3-day period. No diurnal variation in nitrate values was observed. Nitrate concentrations were also measured in cotyledons and unifoliate leaves of 18–21-day-old seedlings of *Melilotus infesta* Guss., an annual sweetclover resistant to sweetclover weevil feeding damage, and cv. Norgold grown in a controlled environment chamber. Nitrate levels in each species fluctuated widely but did not follow a rhythmic or diurnal pattern. There was a significant difference in nitrate levels between the species, with *M. infesta* having significantly higher levels both in cotyledons and in unifoliate leaves (1.65 and 1.58 mg/g, respectively) than Norgold (0.91 and 0.51 mg/g, respectively), and this interspecific difference was more consistent in the early morning.

Keywords: *Nitrate; Melilotus officinalis; Melilotus infesta; diurnal; cotyledon*

INTRODUCTION

The sweetclover weevil, *Sitona cylindricollis* Fahraeus, is the principal insect pest of sweetclover (*Melilotus* spp.) in North America (Beirne, 1971; Craig, 1978). The adult insects leave characteristic crescent-shaped feeding notches on sweetclover leaves and, if present in large numbers, can completely defoliate young plants. Although adult weevils cause damage at all stages of plant growth, the most severe injury occurs at the seedling stage, when high weevil populations can destroy a new stand of sweetclover (Munro et al., 1949; Beirne, 1971).

Manglitz and Gorz (1964) observed that an annual sweetclover, *M. infesta*, was less susceptible to weevil damage than the cultivated biennial species *M. officinalis* and *M. alba*. Analysis of young leaves of *M. infesta* indicated that nitrate levels were 5–10 times higher than in *M. officinalis* leaves (Akeson et al., 1969a,b).

Akeson et al. (1969b) demonstrated that weevil feeding on disks treated with mixtures of *M. infesta* and *M. officinalis* hot water extracts was correlated with feeding predicted from the nitrate content of each mixture. Akeson et al. (1969a,c) also found another unidentified deterrent and a stimulant to weevil feeding in *M. infesta* and *M. officinalis*. The relative contributions of these three factors varied considerably with the stage of development of the plant. Nitrate appeared to be the dominant factor in young leaves and was less important as a deterrent in leaves from mature plants.

Akeson et al. (1969a) and Beland et al. (1970) reported that the nitrate content of the oldest leaves of 6-week-old *M. infesta* plants was 80 times higher than that of very young, "tightly pinched" leaves. Akeson et al. (1969a) observed that feeding damage by the adult sweetclover weevil was correlated with the feeding predicted on the basis of the nitrate content. However, these observations were made on older plants that are more able to withstand defoliation and thus are less sensitive to sweetclover weevil infestations. The nitrate content of the leaves declined with the age of the plant, but mature *M. infesta* plants were resistant to weevil feeding, which would indicate that some factor other than nitrate may be responsible for the feeding resistance in mature plants.

Species, stage of maturity, and soil nitrogen level are important factors influencing nitrate accumulation in plants (Smith and Sund, 1965; Cantliffe, 1973; Brevedan et al., 1977; Sunderman et al., 1979; Iversen et al., 1985). Other factors that influence nitrate concentration include the part of the plant (Crawford et al., 1961), light intensity (Crawford et al., 1961; Chen and Ries, 1969; Steingrover et al., 1986), and soil moisture (Crawford et al., 1961). Since plants are exposed to diurnal cycles of light and temperature, we could expect that the nitrate content of sweetclover seedlings might vary appreciably over a period of a few hours and possibly exhibit a diurnal variation and thus create a window of lower nitrate levels during which the weevils might be expected to feed.

Day-to-day and diurnal variations within a 24-h period in nitrate concentrations have been observed in some plant species (Hageman et al., 1961; Minotti and Stankey, 1973; Hansen, 1980; Gebauer et al., 1984; Iversen et al., 1985; Steingrover et al., 1986) but not in others (Youngberg et al., 1972; Steer, 1974; Iversen et al., 1985; Ferrario et al., 1992). In species with a diurnal cycle, nitrate concentration tends to be high on cloudy days and at the end of the dark period (Iversen et al., 1985; Steingrover et al., 1986). Light quality and intensity and its duration (Harper and Paulsen, 1968; Iversen et al., 1985) affect both the uptake and assimilation of nitrate in the plant (Hansen, 1980; Kallio et al., 1984; Pan et al., 1987; Ferrario et al., 1992; LeBot and Kirkby, 1992). M. D. Rumbaugh (USDA/ARS, Forest and Range Research Laboratory, Utah State University, personal communication, 1991) observed higher nitrate levels in mature sweetclover plants in mid-morning compared to 1 h prior to dawn.

Previous nitrate studies on sweetclover required mature whole plants or plant parts and have not dealt with young growth stages such as seedlings or cotyledons, because the methods were not suited to detect nitrate levels in very small samples. Recent results in our laboratory demonstrated that nitrate levels in

* Author to whom correspondence should be addressed [fax (306) 956-7247; Internet MUIRA@EM.AGR.CA].

† Contribution 1122 Saskatoon Research Centre.

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samples as small as a single cotyledon or trifoliate leaf could be analyzed by HPLC ion chromatography (Muir and Soroka, 1992). With this method, sufficient plant samples can be collected in a short time period to study diurnal or day-to-day variation, as well as changes during the day.

Since sweetclover weevils feed primarily during the night (Munro et al., 1949), the present study was undertaken to determine if diurnal patterns in nitrate levels could be demonstrated in seedlings of *M. infesta* and *M. officinalis* at a stage of growth when sweetclover is very susceptible to sweetclover weevil damage.

MATERIALS AND METHODS

Plant Material. In the field, *M. officinalis* cv. Norgold was seeded in field plots on July 7, 1992, in five rows, 3.0 m long, with 30-cm row spacing in a Sutherland clay loam soil at the Saskatoon Research Centre Farm. No addtional fertilizer was applied. The collection of individual cotyledons ($n = 10$) was started 8 days later, and plants were sampled a total of 18 times starting at 12:00 midnight July 15 and every 4 h thereafter for 3 consecutive days. Twelve cotyledons were sampled randomly over the entire plot area (with a pair of forceps), and the highest and lowest weights were discarded to give $n = 10$ for nitrate analysis. Light intensity was not measured, but cloud cover, temperature, and rainfall were noted.

Controlled environment chamber trials were conducted with Norgold and *M. infesta* seedlings grown in flats in soil-less potting mix consisting of peat, vermiculite, and sand fortified with a controlled release fertilizer (Osmocote 18:6:12; Sierra Chemical, Milpitas, CA) (35 mg/flat), superphosphate, trace elements, and calcium carbonate to control the pH. The flats were maintained in a greenhouse for 15 days after planting and then transferred to a growth chamber where the temperature was kept constant at 17 °C and a 12 h day/12 h night cycle was established under fluorescent and incandescent lights with a light intensity of 18 000–20 000 lx. The seedlings were adapted to these conditions in the growth chamber for 3 days, prior to initiation of sampling. Individual cotyledons and unifoliate leaves from both species and the first trifoliate leaves of Norgold ($n = 10$) were collected 18 days after seeding, and plants were sampled starting at 8:00 a.m. September 14 and every 4 h thereafter for 3 consecutive days (a total of 18 times). Twelve cotyledons or unifoliate leaves or trifoliate leaves were sampled randomly, and the highest and lowest weights were discarded to give $n = 10$ for nitrate analysis.

Nitrate Determination. The cotyledons, unifoliate leaves, and trifoliate leaves were placed in 16-mL polypropylene centrifuge tubes, and fresh weights (FW) were determined within 30–40 min for field samples and within 20–30 min for growth cabinet samples. The extraction solution (2 mL of borate–gluconate HPLC eluent buffer) was then added to each tube (Muir and Soroka, 1992). The excised cotyledons, unifoliate leaves, and trifoliate leaves were crushed individually with a Teflon tissue grinder, allowed to extract for 1 h, and centrifuged at 25000*g* for 15 min, and the supernatant was recovered for nitrate analysis.

A Millipore-Waters liquid chromatograph [Millipore (Canada) Ltd., Mississauga, ON] equipped with a M600E gradient module, M700 autosampler, and 991 photo diode array detector was used to detect nitrate levels (Muir and Soroka, 1992).

The data were analyzed using the General Linear Models analysis of variance procedure with Contrast (SAS, 1989).

RESULTS AND DISCUSSION

Nitrate concentrations in cotyledons of 8–10-day-old field-grown seedlings of *M. officinalis* cv. Norgold were assessed over a 3-day period by HPLC ion chromatography (Figure 1). The nitrate levels varied from 1.01 to 2.83 mg of NO_3/g of fresh weight (FW) and did not exhibit any diurnal variation. The variation observed

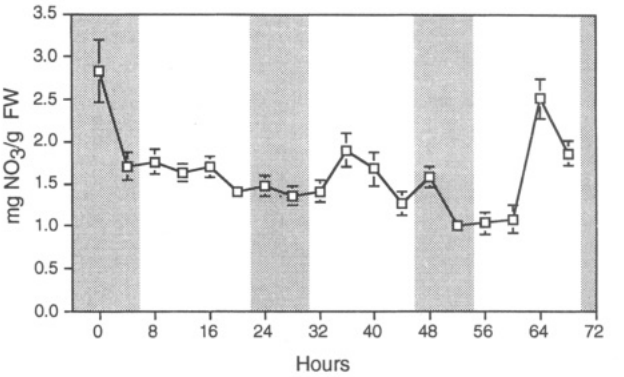

Figure 1. Nitrate concentrations of cotyledons ($n = 10$) from field-grown seedlings of *M. officinalis* cv. Norgold during a 3-day period. Shaded areas represent dark periods. Starting time (0 h) was at 12:00 midnight. When not shown, the SE is within the symbol.

Table 1. Mean (±SE) Nitrate Levels in Cotyledons of *M. officinalis* Cv. Norgold Seedlings Grown for 8–10 Days in the Field

	M. officinalis cv. Norgold
cotyledon ($n = 180$)	
fresh wt, mg	9.80 (±0.50)
nitrate, μg/cotyledon	14.67 (±0.42)
nitrate, mg/g FW cotyledon	1.62 (±0.05)

was within a narrow range, even though environmental factors were changing constantly. In contrast, Minotti and Stankey (1973) noted that environmental variables caused significant rapid changes in the nitrate concentrations of plants growing in the field. The effects of time of collection and the collection day were not significant ($n = 180$) for the weights of the cotyledons, micrograms of NO_3 per cotyledon or milligrams of NO_3 per gram of FW (Table 1). Contrast was used in the analysis of variance, and no significant effect was found on any of the designs. The nitrate concentrations in the cotyledons varied over the 3 days, possibly because the plants were growing rapidly and were utilizing nitrate at different rates depending on their growth patterns (Le Bot and Kirkby, 1992). Weevil feeding damage can be extensive at this stage of sweetclover development, depending on the population of sweetclover weevils present (Craig, 1978).

In the field, there are many environmental factors that also have an effect on the nitrate levels, but no one factor (e.g. temperature, hours of sunshine, temperature and/or growing degree days, wind speed, relative humidity) could be correlated directly to the nitrate levels. In parallel field experiments where ammonium nitrate was incorporated into the soil prior to seeding, there was a possitive correlation between applied fertilizer levels and nitrate levels observed in the cotyledons (Soroka and Muir, 1995), but no impact on weevil feeding was observed, indicating that soil fertility can influence cotyledon nitrate levels.

A similar study was carried out in a growth chamber, where many of these environmental factors could be controlled. Nitrate levels in cotyledons and leaves of 18–21-day-old seedlings of the annual *M. infesta* and biennial sweetclover Norgold were determined (Figure 2). There was a significant difference ($P < 0.01$, $n = 720$) in nitrate levels between the two species (Norgold and *M. infesta*) and also a significant difference ($P < 0.01$, $n = 720$) between the different leaf types (cotyledons, unifoliate leaves, and trifoliate leaves). There was also a significant variety × leaf interaction resulting

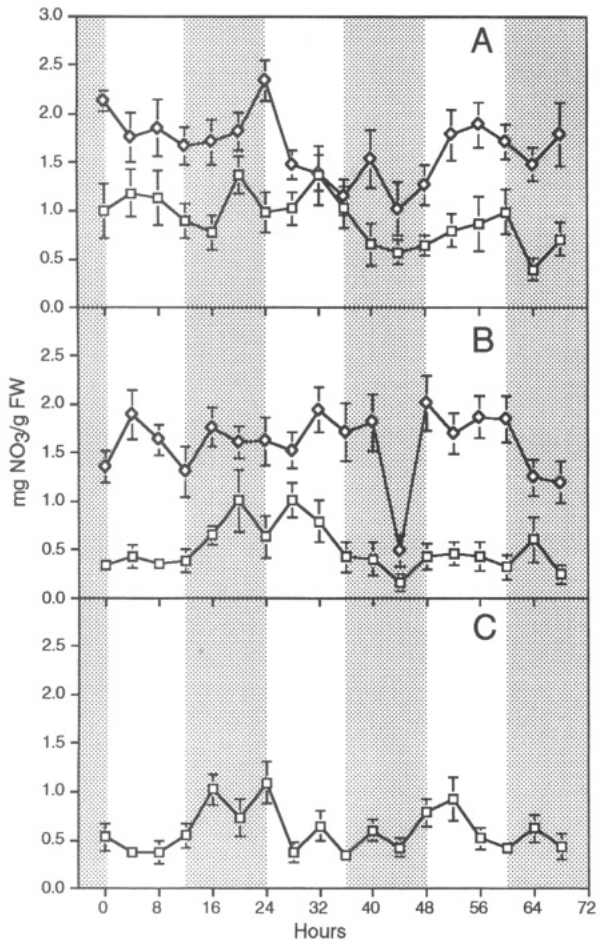

Figure 2. Variations of nitrate concentrations in cotyledons (A), unifoliate leaves (B) of *M. officinalis* cv. Norgold (□) and *M. infesta* (◊) sweetclover seedlings, and trifoliate leaves (C) of *M. officinalis* cv. Norgold, grown in a growth chamber. Shaded areas represent dark periods. Each point represents a mean of 10 replicates or samples, and error bars (SE) are associated with the means. When not shown, the SE is within the symbol. Starting time (0 h) was at 8:00 a.m.

from a larger difference between Norgold cotyledons and Norgold unifoliate leaves than between *M. infesta* cotyledons and *M. infesta* unifoliate leaves.

For the whole experiment, the mean levels of nitrate for *M. infesta* cotyledons were significantly higher ($P < 0.01$, $n = 360$) than those of Norgold cotyledons. The collection day ($n = 360$), the hour of collection ($n = 360$), and the species × hour and species × day interactions for cotyledons were not significant, indicating that in a mixed population the species effect was not significant. When analyzed individually, the day effect and hour effect for the cotyledons of *M. infesta* and Norgold were not significant ($n = 180$), indicating no diurnal effect.

When the nitrate levels in the unifoliate leaves were examined, a significant difference ($P < 0.01$, $n = 360$) between species was observed at all of the times studied. The time of collection ($n = 360$) and the day collected ($n = 360$) were not significant within either species. Within *M. infesta* and Norgold unifoliate leaf data only, there was no significant difference in the hour of collection ($n = 180$) or the day collected ($n = 180$).

The Norgold trifoliate leaves had nitrate concentrations ranging from 0.34 to 1.09 mg/g of FW (Figure 2C), with hour of collection and day collected also being not significant ($n = 180$).

When nitrate values were averaged over the 3 days

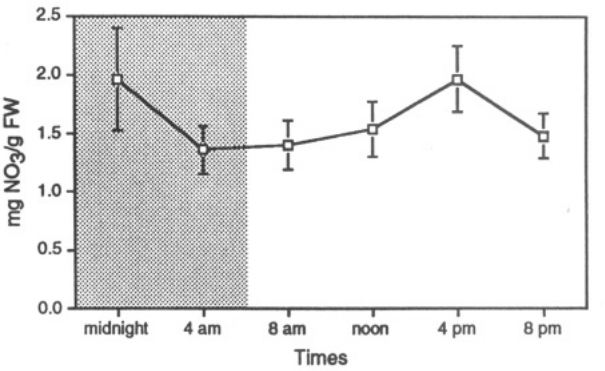

Figure 3. Nitrate levels averaged over 3 days for cotyledons of field-grown *M. officinalis* cv. Norgold sweetclover seedlings. Each point represents a mean of 30 samples, and error bars (SE) are associated with the means. The night period is indicated by shading.

of the experiment, there were small diurnal differences, but these differences were not consistent among plant parts or species. There was a significant difference ($P < 0.01$, $n = 30$) in nitrate concentrations of field-grown seedlings between samples collected at 4:00 p.m. and those collected at 4:00 a.m. (Figure 3). In the growth chamber experiment, there were significant differences in the nitrate values for both cotyledons and unifoliate leaves at different times of the day within each species (Figure 4A,B). The results of the growth chamber experiment indicate that, although no single collection time consistently gave the lowest sampling error, dawn or early morning may be the best time to collect plant material when selecting for high nitrate accumulation in *Melilotus* species. At this time, the differences between species were consistently at their maximum for cotyledons and unifoliate leaves (Figure 2). Within each species, and plant part, the lack of large variations in sampling error over the range of sample times suggested that there is no particular time in which variation within a species would be at a maximum.

The mean nitrate levels measured in the cotyledons of *M. officinalis* cv. Norgold seedlings grown in the field were higher than in seedlings grown in the growth chamber (Tables 1 and 2), although the range of values was approximately the same. In previous work, we demonstrated that cotyledons from 7-day-old Norgold seedlings grown in the greenhouse contained significantly more nitrate (2.83 mg/g) than those of *M. infesta* seedlings (1.70 mg/g) (Muir and Soroka, 1992). By 21 days, it was observed that the nitrate levels in all parts of *M. infesta* seedlings were significantly higher than those of corresponding Norgold seedlings. In samples collected in the early morning from 1993 field-grown 21–23-day-old seedlings, *M. infesta* cotyledons and unifoliate and trifoliate leaves (3.32, 2.38, and 2.26 mg/g, respectively) contained significantly ($P < 0.01$, $n = 30$ for each case) more nitrate than Norgold cotyledons and unifoliate and trifoliate leaves (1.60, 1.25, and 1.67 mg/g, respectively). In the growth chamber experiment, the nitrate concentrations in the cotyledons and unifoliate leaves of *M. infesta* were also significantly higher than those of *M. officinalis* cv. Norgold at 18–21 days postseeding (Table 2). Since *M. infesta* accumulates more nitrate overall in the leaves at this stage (18–23 days), this consistent difference between these two species in nitrate metabolism may be attributed to the genetic characteristics of each of these species.

While it is possible that there is sufficient micro-environmental variability in soil nitrate levels to ac-

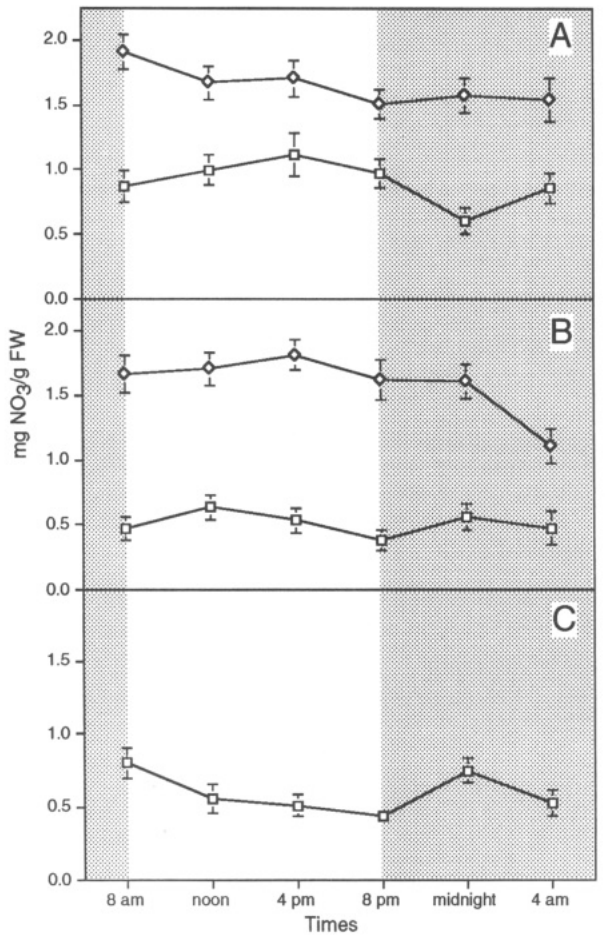

Figure 4. Nitrate levels averaged over 3 days in cotyledons (A), unifoliate leaves (B) of *M. officinalis* cv. Norgold (□) and *M. infesta* (◊) sweetclover seedlings, and trifoliate leaves (C) of *M. officinalis* cv. Norgold, grown in a growth chamber. Each point represents a mean of 30 samples collected, and error bars (SE) are associated with the means. When not shown, the SE is within the symbol. The night period is indicated by shading.

Table 2. Mean (±SE) Nitrate Levels in 18–21-Day-Old Seedlings of *M. officinalis* Cv. Norgold and *M. infesta* Grown in a Growth Chamber

	M. officinalis cv. Norgold	*M. infesta*
cotyledon (n = 180)		
fresh wt, mg	14.00 (±0.20)	21.30 (±0.30)
nitrate, μg/cotyledon	12.90 (±0.76)	35.33 (±1.31)
nitrate, mg/g FW cotyledon	0.91 (±0.05)	1.65 (±0.06)
unifoliate leaf (n = 180)		
fresh wt, mg	35.60 (±0.50)	37.30 (±0.60)
nitrate, μg/leaf	18.17 (±1.46)	61.20 (±2.58)
nitrate, mg/g FW leaf	0.51 (±0.04)	1.58 (±0.06)
trifoliate leaf (n = 180)		
fresh wt, mg	16.60 (±0.40)	
nitrate, μg/leaf	10.18 (±0.70)	
nitrate, mg/g FW leaf	0.60 (±0.04)	

count for the variability in cotyledon nitrate levels, any diurnal variation in nitrate uptake/metabolism should be greater if diurnal variation is to have any effect on weevil feeding patterns. The inability of sweetclover weevils in feeding trials to perceive any microenvironmentally induced variability in cotyledon nitrate levels (Soroka and Muir, 1995) suggests that this type of variability is not significant.

In conclusion, this study demonstrated that nitrate concentrations in cotyledons of 8–10-day-old field-grown Norgold sweetclover seedlings varied substantially over 3 days but did not display any diurnal variation. This lack of diurnal variation was also observed in cotyledons and unifoliate leaves of 18–21-day-old *M. infesta* and Norgold seedlings, as well as trifoliate leaves of Norgold seedlings grown in a growth chamber. When the whole data set is considered, the most consistent inter-specific difference was observed after dawn, while maximum intraspecific differences were not consistently observed at any time. At this early seedling stage of development in sweetclover, there is a high degree of variability in the nitrate levels in the different plant parts and within the different species, and this variability is not reflected in weevil feeding patterns (Soroka and Muir, 1995). The lack of a distinct diurnal variation in nitrate levels suggests weevil feeding times are not influenced by nitrate levels.

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