FACTORS AFFECTING CONCENTRATIONS OF DIMETHYLATED INDOLEALKYLAMINES IN *PHALARIS TUBEROSA* L.

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Summary

In an experiment conducted in controlled-environment rooms, factors (temperature, light intensity, and nitrate supply) that influenced nitrate nitrogen concentrations in *Phalaris tuberosa* also affected concentrations of tryptamine alkaloids.

Concentrations and yields of tryptamine alkaloids increased with higher day–night temperature regimes in both full sunlight and shade (28% sunlight); concentrations also increased with nitrate supply in full light but not in shade. Shaded plants had higher concentrations of alkaloids than unshaded plants at all levels of nitrate supply. Except for a difference between trends with levels of nitrate supply at 8 and 16 hr light, alkaloid concentrations were not affected by day length.

In field-grown plants dimethyltryptamine, 5-methyoxydimethyltryptamine, and 5-hydroxydimethyltryptamine responded similarly to reductions in light intensity, the former being the dominant indole alkylamine in plants grown in the field and in the phytotron.

I. Introduction

Neurological disorders or sudden deaths among sheep grazing the Mediterranean grass *Phalaris tuberosa* L. have been reported by McDonald (1942, 1946), Lee and Kuchel (1953), Lee (1956), and Moore *et al.* (1961). Following the discovery by Culvenor, Dal Bon, and Smith (1964) that *P. tuberosa* may contain *N*,*N*-dimethylated indolealkylamines, principally *N*,*N*-dimethyltryptamine (DMT), 5-methoxy-*N*,*N*-dimethyltryptamine (5MDMT), and 5-hydroxy-*N*,*N*-dimethyltryptamine (5HDMT), Gallagher *et al.* (1964), by administering small doses (0·1 mg/kg body wt.) of DMT or 5MDMT to sheep, were able to reproduce signs of neurological disorders similar to those evidenced in sheep suffering from acute phalaris staggers. Higher dosages (1·5–2·0 mg/kg body wt.) caused cardiac arrest and frequently were fatal. 5MDMT appeared to be more potent than HDMT or DMT. Pathological and biochemical aspects of the diseases are discussed by Gallagher, Koch, and Hoffman (1965).

The circumstances in which sheep collapsed and died while grazing *P. tuberosa* at Canberra between 1960 and 1965 were described by Moore and Hutchings (1967).

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Deaths appeared to be associated with high levels of nitrate in soil and in pasture, suggesting the possibility of a relationship between nitrate nitrogen and concentrations of tryptamine alkaloids in *P. tuberosa*. Aspects of this relationship were investigated in these present studies by varying the supply of nitrate and other factors affecting nitrate accumulation in plants. Experiments were conducted in the CSIRO phytotron under controlled conditions and at the CSIRO Ginninderra Field Station on phalaris pastures that were toxic to sheep. *P. tuberosa* in these pastures contained tryptamine alkaloids in excess of 40 mg/100 g dry weight. The same cultivar (Australian commercial) growing in nearby grass garden rows without clover or added nitrogen contained only 11 mg/100 g dry weight. One of the aims of the field experiment was to see if reductions in light intensity altered the proportions of the three principal alkaloids, a matter of some interest in view of the seemingly greater potency of 5MDMT to sheep.

II. Experimental and Analytical Methods

(*a*) *Determinations of Tryptamine Alkaloids and of Nitrate Nitrogen*

Grass samples collected in the phytotron or field were placed immediately in containers with dry ice. Alkaloids were extracted from the samples by an adaptation of the procedure used for serotonin by Bogdanski *et al.* (1956) and were then separated by paper chromatography. In the modified procedure of Gallagher, Koch, and Chia (personal communication) fresh grass (3–5 g fresh wt.) was extracted with 15 ml 0·1N HCl, by macerating in a Servall omnimixer. The pH of the macerate was adjusted to about 10 by the addition of anhydrous Na_2CO_3. NaCl (5 g) and 15 ml n-butanol were added and the mixture was shaken for 5 min. After centrifuging the butanol phase was decanted into a clean tube. The aqueous phase was washed twice with 3 ml n-butanol and the washings added to the butanol extract. Then 42 ml n-heptane and 0·5 ml 0·1N HCl were added and the tube was shaken and centrifuged. The acid layer was transferred into a clean, dry, graduated tube, and the butanol–heptane phase was re-extracted twice with 0·5 ml 0·1N HCl.

Aliquots (20%) of the combined acid extract were applied in 2-in. bands to paper chromatograms with standard spots of DMT, 5MDMT, and 5HDMT on the margins and the chromatograms developed overnight (16–18 hr) in butanol–acetic acid–water (12 : 3 : 5 v/v) by the descending method. The separated alkaloid bands were detected and identified by spraying the dried chromatograms with a 0·1% xanthydrol solution in 95% ethanol–5% 10N HCl.

The appropriate bands were placed into test tubes and their alkaloid concentration estimated colorimetrically by a modification of the method of Weissbach *et al.* (1959). The following reagents were added in sequence to the test tube containing the chromatogram band: 1 ml 0·1N HCl, 1·2 ml 10N HCl, and 2·5 ml 1% xanthydrol in glacial acetic acid. After mixing for 10 min, 1·25 ml 5% sodium bisulphite was added and after a further 10 min the coloured liquid was decanted into a tube and centrifuged to sediment the cellulose fibres. The absorbance of the clear supernatant liquid was measured in a spectrophotometer against controls containing standard amounts of DMT (515 mμ) and 5MDMT and 5HDMT (585 mμ).

Standard alkaloid and solutions prepared from blank strips of chromatograms were prepared simultaneously in the same manner to obtain calibration curves from which the alkaloid concentration of the chromatogram band could be read.

Nitrates were determined with brucine by a modification of the method of Horne and Denmead (1955).

(b) *Effects of Environmental Factors on Tryptamine Alkaloid and Nitrate Levels in* P. tuberosa

Plants of the Australian commercial cultivar of *P. tuberosa* raised in perlite under uniform conditions for 7 weeks were subjected to different day lengths and to varying intensities of light, temperature, and nitrate supply in phytotron cabinets exposed to daylight. The experiment was a complete $3\times3\times2\times2$ factorial with nine pots (18 plants) in each treatment. Subsequently it was found necessary to bulk the nine replicates in order to get sufficient plant material for nitrate and alkaloid determinations.

Day lengths were reduced to 8 hr by light-proof covers and increased to 16 hr by low-energy (50 f.c.) incandescent lamps. Light intensity was reduced to 28% full sunlight at noon by screens of plastic gauze. Three levels of nitrate nitrogen were established by watering twice daily with nutrient solutions containing 0·05, 0·5, and 5 times the nitrogen in Hoagland's nutrient solution which had been modified by halving the phosphorus content. Three weeks after plants were cut to ground level and the nitrogen treatments commenced, the regrowth was harvested for determinations of dry weights, nitrates, and tryptamine alkaloids.

(c) *Effects of Artificial Shading on Tryptamine Alkaloid Concentrations in* P. tuberosa *Growing in Pasture Swards*

Frames $12\times8\times3$ ft covered with organdie or different thicknesses of plastic gauze were placed on the pasture to obtain a range of light intensities at grass level. One layer of organdie and one, two, and three layers of plastic gauze reduced light intensities to 33–41, 27–34, 11–17, and 5–10% respectively of full sunlight at noon. The covered frames which were open at the bottom to minimize temperature differences between shaded and unshaded areas were placed at random on a *P. tuberosa* sward on April 14, 1965. There were two replicates of each light treatment. On July 6, grass in the open and in the partially covered frames was sampled, placed in dry ice, and extracted for alkaloids as already described.

III. Results

(a) *Phytotron Experiment*

The treatments applied and their main effects are shown in Table 1. DMT was the dominant alkaloid and as the other two compounds were present in much smaller and more variable amounts treatments were compared by their effects on the combined concentrations of the three alkaloids.

Day length had little effect but temperature, light intensity, and nitrogen supply each influenced dry weights and concentrations of nitrate and tryptamine alkaloids. Concentrations of nitrate increased linearly with temperature and with log nitrate supply. Alkaloid concentrations responded linearly to temperature but irregularly to nitrate supply. The reason for this irregularity was a difference in the response of alkaloids to nitrate supply in full and reduced light. This is shown in Figure 1.

TABLE 1

EFFECTS OF DIFFERENT DAY–NIGHT TEMPERATURE REGIMES, DAY LENGTH, LIGHT INTENSITY, AND NITRATE LEVELS ON DRY WEIGHT, NITRATE NITROGEN, AND TRYPTAMINE ALKALOID CONCENTRATION IN *P. TUBEROSA*

Environmental Factor	Nitrate Nitrogen (p.p.m.)	Tryptamine Alkaloid Concn. (mg/100 g dry wt.)	Dry Weight of 18 Plants (g)
Temperature:			
9°C day— 4°C night	4864	34·3	27·3
15°C day—10°C night	6025	48·5	34·9
21°C day—16°C night	8849	69·3	36·9
Day length:			
8 hr	6334*	49·3*	32·5*
16 hr	6825*	52·0*	33·5*
Light intensity:			
Full sunlight	4084	38·4	40·2
28% sunlight	9075	62·9	25·8
Relative nitrate levels:			
1	3267	49·4*	28·9
10	6645	44·7*	33·8
100	9826	57·9	36·3

* Differences between the two values not significant at 5% level.

Temperature effects on concentrations of nitrate and of alkaloids were similar in both full sunlight and shade (Fig. 2).

(*b*) *Effects of Artificial Shading on Tryptamine Alkaloid Concentrations in* P. tuberosa *in a Pasture Sward*

Concentrations of DMT, 5MDMT, and 5HDMT at the different light intensities are shown in Figure 3. All alkaloids responded similarly to reductions in light intensity. There were marked increases in concentrations between approximately 40 and 12% full sunlight followed by a decline below 10% full light.

IV. DISCUSSION

The factors temperature, shade, and nitrate supply that increased nitrate nitrogen levels in *P. tuberosa* also increased tryptamine alkaloid concentrations. Mean concentrations of plant nitrate and alkaloids were not affected by day lengths (Table 1) but there was a significant interaction between day length and nitrate supply on alkaloid concentration. Most of this interaction is attributable to a difference between trends of alkaloid concentrations with increasing levels of nitrate supply at day lengths of 8 and 16 hr. At 8 hr the trend was linear; 39·3, 48·1, and 60·5 mg per 100 g at relative nitrate levels of 1, 10, and 100 respectively. At 16 hr the corresponding concentrations were 59·0, 41·3, and 55·2 mg per 100 g. It is not clear why lengthening the light period to 16 hr should increase alkaloid concentrations at the lowest but not at higher levels of nitrate.

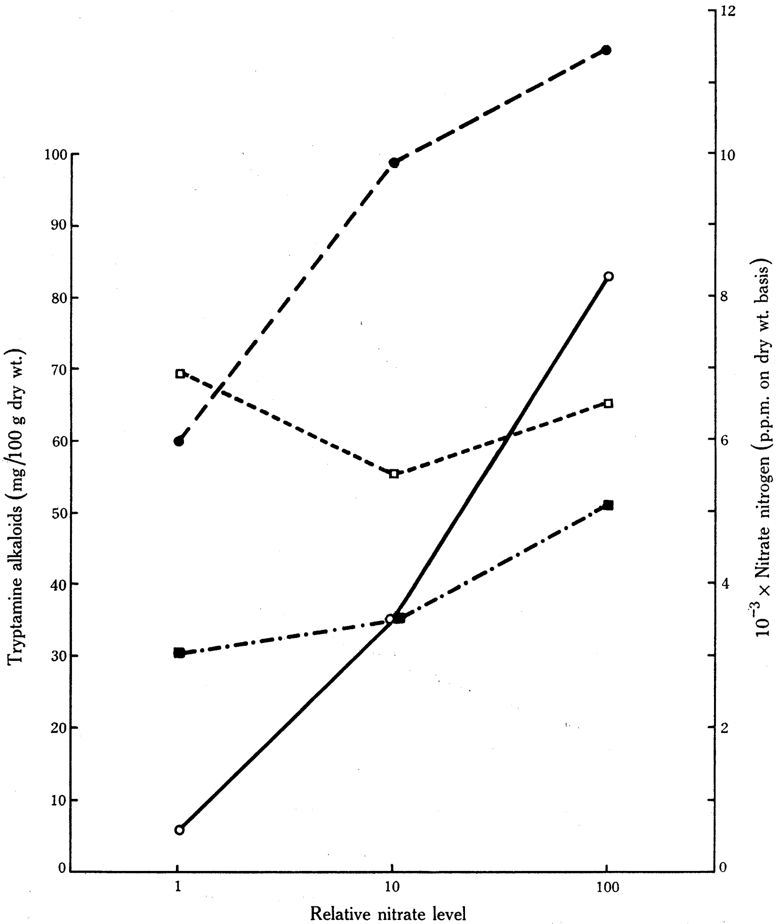

Fig. 1.—Effects of nitrate supply on concentrations in full sunlight and in shade (28% full sunlight) respectively of tryptamine alkaloids (■, □) and of nitrate nitrogen (○, ●) in *P. tuberosa*. Means of three temperature and two day-length treatments.

Responses of nitrate and of alkaloids to temperature were similar; each increased in concentration with air temperatures both in full light and in shade. Both were higher in shaded plants and increased more steeply from the medium to the highest temperature regime in shaded than unshaded plants (Fig. 2), presumably because of a decline in the dry matter production of shaded plants at the highest temperature regime. Nitrate nitrogen levels in *P. tuberosa* increased also with nitrate supply in full sunlight and in shade and were higher in shaded plants at all levels of supply. Alkaloids were high (nearly 70 mg/100 g) in shaded plants even at the lowest level of nitrate supply but, unlike plant nitrate, did not respond to increases in nitrate supply. In full light, however, alkaloid concentrations increased in an approximately linear fashion with log nitrate supply (Fig. 1).

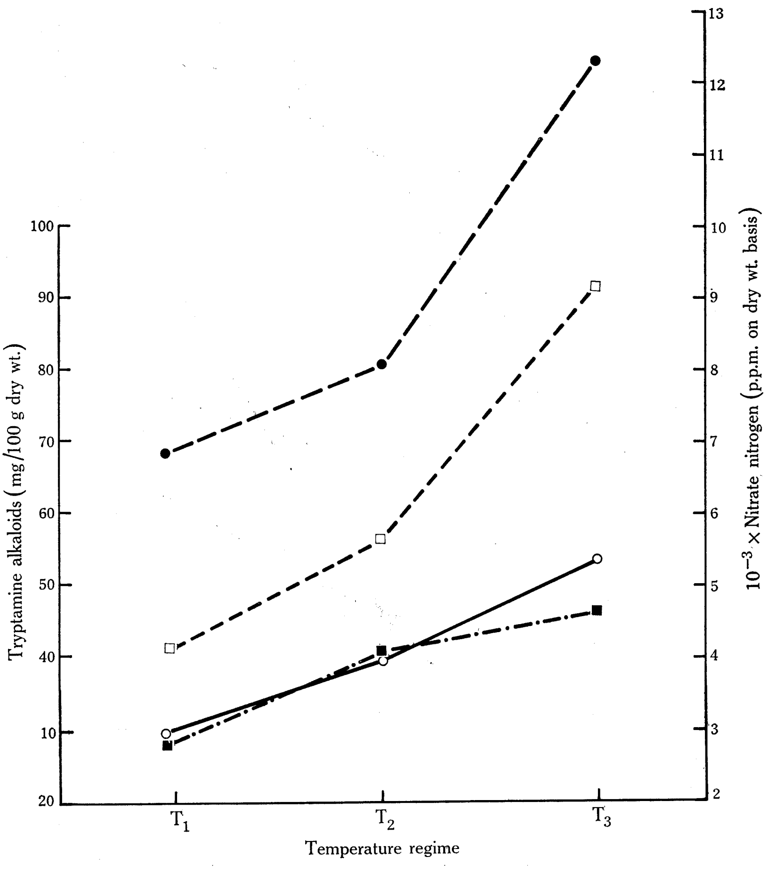

Fig. 2.—Effects of three day–night temperature regimes, T_1 (9–4°C), T_2 (15–10°C), and T_3 (21–16°C) on concentrations of nitrate nitrogen (○, ●) and tryptamine alkaloids (■, □) in *P. tuberosa* grown in full sunlight and in 28% full sunlight, respectively. Means of three nitrate and two day-length treatments.

The interaction between light intensity and nitrate supply was significant for both plant nitrate and alkaloid concentrations. From the lowest to the highest nitrate level alkaloid concentrations increased 1·6-fold in full light but fell slightly in reduced light. The proportionate increase in plant nitrate from the lowest to the highest level was several times greater in full than in reduced light.

Yields of nitrate nitrogen per plant were 42% higher in shaded than unshaded plants but alkaloid yields per plant were scarcely affected by reduction in light intensity. Mean yields of alkaloids were 16·2 and 15·4 mg for the shade and full sunlight treatments respectively, suggesting that the higher concentrations in shaded plants are due to their lower production of dry matter in comparison with plants grown in full sunlight.

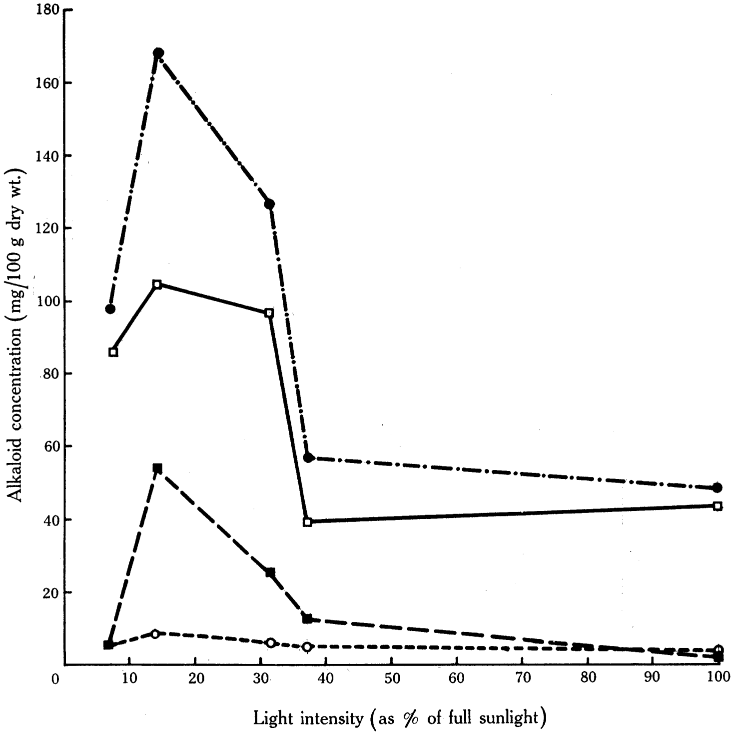

Fig. 3.—Effects of artificial shading on concentrations of dimethyltryptamine (□), 5-methoxydimethyltryptamine (■), and 5-hydroxydimethyltryptamine (○) in sward-grown *P. tuberosa*. ● Total alkaloids. Means of two replicates.

Alkaloid yields per plant increased with temperature and with nitrate supply (Fig. 4). The response of alkaloid yield to temperature, pooled for light and shade plants, is linear. This reflects concurrent increases in alkaloid concentrations and in dry matter as temperatures are raised (Table 1).

The effects of temperature on alkaloid yields were similar in light and in shade. Mean yields of alkaloids from unshaded plants were 8·55, 16·81, and 22·81 mg at the low, medium, and high temperature regimes respectively. Corresponding yields from shaded plants were 9·71, 16·05, and 22·83 mg (each value is a mean of 12 groups of 18 plants). The nitrate supply curve reflects the different responses of alkaloid concentrations in shaded and unshaded plants to log nitrate supply. Figure 5 shows the yields of alkaloids from light and shade plants at three levels of nitrate supply.

Alkaloid yield response curves resemble the concentration curves (Fig. 1) suggesting that nitrate supply influences alkaloid biosynthesis in full sunlight, that is, when nitrate concentration in the plant is otherwise low. In reduced light, plant nitrate concentrations are high even at low levels of supply and alkaloid yields are not increased by raising the amount of nitrate nitrogen in the growth medium.

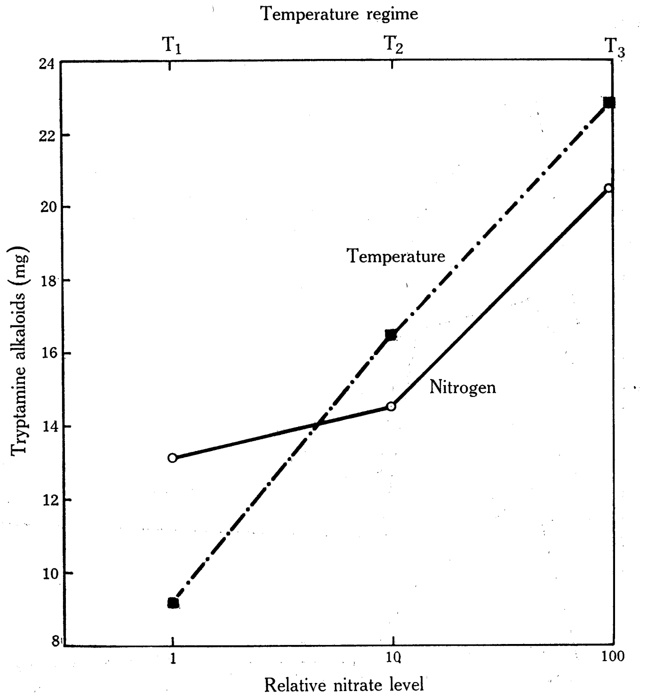

Fig. 4.—Yields of tryptamine alkaloids from *P. tuberosa* grown at three day–night temperature regimes, T_1 (9–4°C), T_2 (15–10°C), and T_3 (21–16°C), and at three levels of nitrate supply. Means of three temperature or three nitrate and two day-length and two light intensity treatments.

As in the phytotron experiment, DMT was the dominant alkaloid in sward-grown *P. tuberosa*. The other two tryptamine alkaloids were present in low concentrations except at 12% full sunlight when 5MDMT rose to a concentration of more than 50 mg per 100 g dry weight. In view of its relatively high toxicity this compound could be a factor of some significance in the poisoning of sheep on rested pastures in which there is a high degree of inter- and intraplant shading or in which there is a large carryover of dead material from the previous growing season. The decline in alkaloid concentration below 10% full light suggests the possibility that carbohydrate may have become limiting for alkaloid synthesis.

The quantity of tryptamine alkaloids available is an important factor in the health of grazing animals and depends on the concentration of the alkaloid and the amount of grass present. Toxicity may result from ingestion of a relatively small amount of grass with a high alkaloid content or a large amount with a relatively low content.

The results of experiments in the phytotron (Figs. 4 and 5) and the field (Fig. 3) suggest that *P. tuberosa* which has been subjected to very low light intensities for some time is likely to be toxic to sheep in all circumstances. But if light intensities are high the grass is unlikely to be toxic unless soil nitrate levels also are high. Danger to animals seemingly increases when day temperatures are 20°C or above.

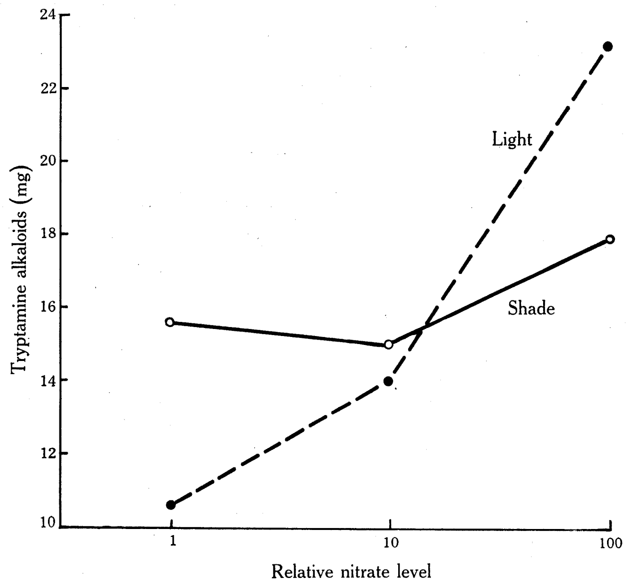

Fig. 5.—Yields of tryptamine alkaloids from *P. tuberosa* grown in full light and in shade (28% full sunlight) at three levels of nitrate supply. Means of three temperature and two day-length treatments.

The experimental findings are seemingly in accord with field observations on the occurrences of sudden deaths among sheep. Moore *et al.* (1961) and Moore and Hutchings (1967) have reported numerous sudden deaths among sheep grazing on phalaris growing in soils which have accumulated relatively high levels of nitrogen as a result of several years of clover growth. Deaths in southern Australia usually occurred in the autumn period when soil mineralization processes are active. It was also observed that deaths often occurred on pastures in which young shoots of phalaris were partially shaded by dead litter from a previous season's growth, i.e. on lightly stocked pastures. Autumn in southern Australia is a period of falling rather than rising temperatures, but average daily maximum temperatures at Canberra are 20 and 15°C for April and May, respectively. The phytotron data indicate that these temperatures favour high alkaloid concentrations, particularly when light intensities are low.

The high alkaloid concentrations at warm temperatures stress the desirability of using low alkaloid cultivars of *P. tuberosa* (Oram and Williams 1967) in *P. tuberosa* × *P. arundinacea* L. hybrids which have promise for summer as well as winter production in southern Australia.

V. References

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