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Effects of cutting frequency and height on alkaloid production in endophyte-infected drunken horse grass (*Achnatherum inebrians*)

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An evaluation was performed on the influence of mowing height (2.5, 5.0 or 7.5 cm) and mowing frequency (weekly or fortnightly) on levels of ergot alkaloids (ergine and ergonovine) formed in drunken horse grass, *Achnatherum inebrians*, grown under greenhouse conditions. Samples were taken monthly and alkaloids were extracted and analyzed by reverse-phase HPLC. Alkaloid levels increased more or less linearly with plant age over the first four months following establishment. Levels were higher in samples cut fortnightly compared to those cut weekly, and were higher when plants were cut at a mowing height of 7.5 cm vs. 2.5 cm. In most cases, the highest alkaloid levels observed were almost three times those of the lowest. If plant protection applications are developed for the endophytic fungus, *Neotyphodium gansuense*, it will be necessary to be aware of the potential role of plant husbandry practices (e.g., defoliation frequency and intensity) for reducing or enhancing levels of plant alkaloids. Similar husbandry factors may affect alkaloid levels in other *Neotyphodium*-grass associations, which would be interesting for further study.

***Neotyphodium gansuense*, *Achnatherum inebrians*, mowing height, mowing frequency, ergine, ergonovine**

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Endophytic fungi belonging to the related genera *Epichloë* and *Neotyphodium* have been found in many cool-season grasses (subfamily Pooideae) [1,2]. Previous studies have focused mainly on the endophytes of *Lolium* and *Festuca*, because alkaloids produced in these associations are toxic to grazing animals [3–8].

Drunken horse grass (DHG, *Achnatherum inebrians*) is another endophyte-infected grass reported to be toxic to livestock. In China, DHG is mainly distributed throughout arid, semi-arid, alpine and subalpine native grasslands of Gansu, Xinjiang Uyghur Autonomous Region, Qinghai and Ningxia Hui Autonomous Region as well as Inner Mongolia and Tibet [9]. Toxicity symptoms are typically seen in livestock after grazing DHG when forage is in short supply, which are apparently caused by the endophyte *Neotyphodium gansuense* [10,11]. However, the *N. gansuense* endophyte provides DHG with a strong competitive ability due to an increased host tolerance to abiotic [11] and biotic [12–14] stresses. Endophyte-infected (E+) DHG has been shown to contain high levels of the ergot alkaloids, ergine and ergonovine [15,16].

A significant research need is better understanding of factors affecting alkaloid levels in grass-endophyte associations. In turf culture, there exists an interest in formulating husbandry practices that enhance alkaloid levels, as higher levels typically discourage predators [17–20]. By contrast, such information can be useful to pastoralists in the formulation of husbandry practices that minimize the exposure of grazing animals to these toxins. One point that has received comparatively little study is the finding described by

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Salminen and Grewal [21] and Salminen *et al*. [22] that an increase in defoliation frequency or intensity decreases the levels of some alkaloids produced by *Neotyphodium lolii* and *Neotyphodium coenophialum* in perennial ryegrass and tall fescue, respectively. The findings of these two studies might represent a general response of grass-endophyte associations. To test this hypothesis, the following experiments were performed using a different grass-endophyte association, DHG and *N. gansuense*, in China.

1 Materials and methods

To determine whether the effects of cutting height and frequency previously reported represent a more general phenomenon, we largely followed previous experimental methodology [21,22] with minor modification.

1.1 Plant establishment

DHG seeds were collected in September 2005 from endophyte-infected plants grown in the experimental area of the College of Pastoral Agricultural Science and Technology, Lanzhou University, Lanzhou, China. Pots (30 cm×25 cm×8 cm) were filled with silt loam soil sterilized in an oven at 160°C for 6 h. Only well-filled, healthy-looking seeds were used. Five rows of 10 seeds were planted in each pot. Pots were placed in a constant temperature greenhouse (22°C) with a day/night cycle of 16 h light/8 h dark, using artificial light, and were watered as required. Seedlings emerged approximately 3–4 d after planting. From approximately one month onward, plants were defoliated weekly to 5 cm above soil level.

1.2 Cutting height

At approximately six weeks from sowing, 36 pots (3 replicates×3 treatments×4 harvest dates) were randomly assigned to three cutting height treatments (2.5, 5.0 or 7.5 cm above the soil surface). Plants were cut weekly to the designated height for one, two, three or four months. Nine pots in each time interval were destructively harvested by cutting to ground level approximately a quarter of the herbage in the center of the pot. These samples were immediately frozen in liquid nitrogen, lyophilized, ground to a powder and then stored for analysis.

1.3 Cutting frequency

To investigate effects of cutting frequency on alkaloid levels, 24 pots (3 replicates×2 treatments×4 harvest dates) were randomly assigned to two cutting treatments: Either plants were cut weekly, or once every two weeks. The cutting height was 5.0 cm above the soil surface. Sample collection from the center of each pot was carried out monthly, over four months, as described above.

1.4 Alkaloid extraction

The freeze-dried and ground grass material (50 mg) was extracted in two stages using a two-phase solvent system, following a method modified from Miles *et al*. [15]. These samples were quantified on an Agilent 1100 HPLC, with an Agilent 250 mm×4.6 mm C_{18} column containing 5 μL particles. Mobile phases were "A" (0.1 mol L^{-1} NH_4OAc) and "B" (CH_3CN∶0.1 mol L^{-1} NH_4OAc, 3∶1) used at a flow rate of 1 mL min^{-1}. The elution scheme was 95% A (initial 10 min), then ramped down over 10 min to 80% A, then ramped down over 10 min to 50% A, and finally ramped back up over 5 min to 95% A. A fixed-wavelength UV detector (G1314A VWD, Agilent) set at 312 nm was used to detect the alkaloids. Alkaloid levels in 20 μL injection samples were quantified by comparing their peak areas with external standard curves. Ergonovine was purchased from Sigma-Aldrich China. The ergine used was a gift from Dr. Miroslav Flieger, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

1.5 Statistical analysis

Variation in ergot alkaloid levels at different mowing heights and frequencies was analyzed using a factorial analysis of variance (ANOVA) procedure (SPSS 13.0, Chicago, IL, USA), using pots as experimental units. The ANOVA included a term for the treatment and time interactions.

2 Results

Results were consistent across replicates with small measurement errors compared to the size of treatment effects. This led to statistically significant results (P<0.001) for both of the endophytic alkaloids and for all treatment effects and interactions for which F-tests were performed.

2.1 Cutting height

All effects of the repeated measures ANOVA are shown in Figure 1. Levels of ergonovine were significantly (P<0.05) higher than levels of ergine in all parts of E+ DHG plants. In general, ergonovine levels were about double those of ergine. Levels of both alkaloids increased over the growing months and levels increased with plant age and with cutting height, in a nearly linear pattern.

The effects of plant cutting heights on ergine levels were significant (P<0.05), although the difference between 2.5 and 5.0 cm was not significant (P>0.05) at three months. All the results among months were also significant (P<0.05). There was an interaction between plant age and defoliation height, in that the cutting height effect was smaller in plants at one month of age (Figure 1A).

The effects of cutting height on ergonovine levels were also significant (P<0.05), although the difference between 2.5 and 5.0 cm was not significant (P>0.05) after cutting at one month, and the difference between 5.0 and 7.5 cm was not significant (P>0.05) at four months. All the results among months were also significant (P<0.05) (Figure 1B).

2.2 Cutting frequency

The effects of cutting frequency closely paralleled those of cutting height, with initial levels of the two alkaloids being similar and increasing in a similar way with plant age. However, the rise in endophytic alkaloid levels was greater with less frequent defoliation than those with increased cutting height, with differences in levels between frequently and infrequently cut plants continuing to widen throughout the experiment.

The effects of cutting frequency and month of growth on ergine levels were significant (P<0.05). There was significantly (P<0.05) more ergine in plants cut weekly than those biweekly at two, three, and four months, with increases of 45.65%, 59.6% and 68.2%, respectively (Figure 2A).

The effects of cutting frequency and month of growth on ergonovine levels were also significant (P<0.05). There was significantly (P<0.05) more ergonovine in plants cut weekly than those biweekly at two, three, and four months, with increases of 28.6%, 31.9% and 35.1%, respectively (Figure 2B).

3 Discussion

The toxicity of DHG to *Equus caballus*, *E. asinus* and *E. caballus*×*E. asinus* has been reported by Dang *et al*. [23]. Miles *et al*. [15] reported that ergonovine and lysergic acid amide (i.e., ergine) were the major ergot alkaloids in DHG in Xinjiang Uyghur Autonomous Region. Li *et al*. [16] reported ergonovine and ergine levels and their temporal variation in *Neotyphodium* endophyte-infected (E+) and endophyte-free (E−) DHG grown in Gansu Province. Zhang and Chu [24] isolated ergonovine (25 mg from 2 kg) and ergonovinine (30 mg from 7 kg) from dry powdered DHG. Sang *et al*. [25] also detected seven alkaloids from DHG grown near the Jinqiang River, Gansu Province. The steps in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass have been reported by Wang *et al*. [26].

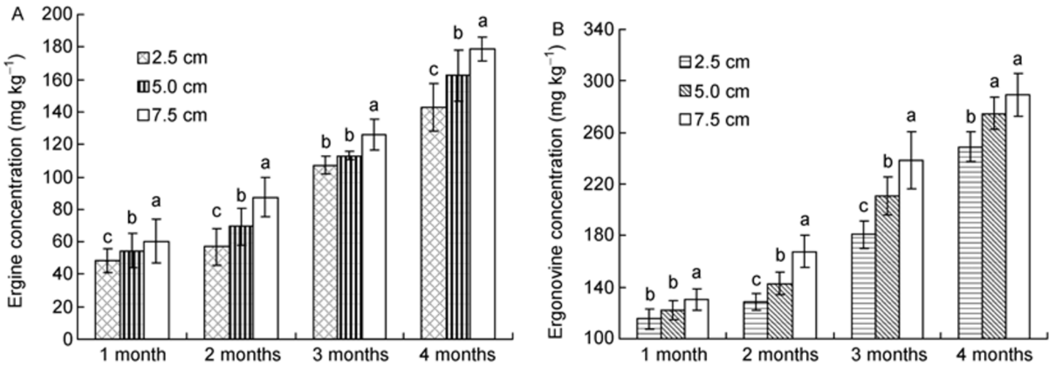

Figure 1 Effect of cutting weekly at different heights (2.5, 5.0 or 7.5 cm) on ergot alkaloids (A for ergine and B for ergonovine) concentrations in endophyte-infected drunken horse grass (*Achnatherum inebrians*). Values are means±standard error (SE) and bars indicate SE. Non-matching small letters indicate a significant difference (P<0.05).

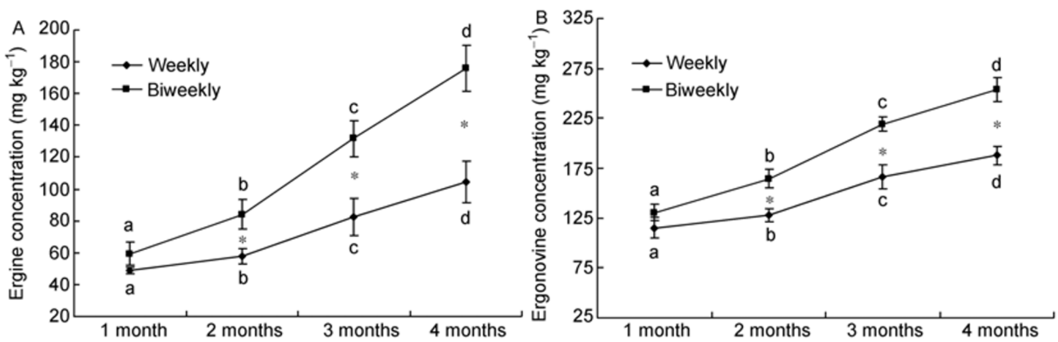

Figure 2 Effect of different cutting frequency (weekly or biweekly) on ergot alkaloids (A for ergine and B for ergonovine) concentrations in endophyte-infected drunken horse grass (*Achnatherum inebrians*). Values are means±standard error (SE) and bars indicate SE. * shows a significant difference (P<0.05) between mowing frequencies. Non-matching small letters in the same line indicate significant difference (P<0.05).

It has been shown that alkaloid levels in both the leaf blades and seed ears were significantly ($P<0.05$) higher than those in stems [16]. In accordance with the distribution of the endophyte in plant tissues, alkaloids were concentrated within the more terrestrial sections (rather than the aerial parts) of the plants. The same trend has been observed for peramine and Lolitrem B in infected perennial ryegrass, with the highest levels being in the crown near the soil; 75% of the alkaloids are found within 5 cm of the most basal parts, with the older leaves having higher alkaloid levels vs. young leaves [27–29]. By contrast, levels of ergot alkaloids have been reported highest in the seeds of infected tall fescue, significantly higher than in other plant parts [30–32].

Biweekly cutting has been shown to result in higher levels of ergovaline, ergonovine, and ergocristine in endophyte-infected tall fescue, with ergocristine and two putative alkaloids following a similar pattern in perennial ryegrass [21]. We found these same general patterns for ergine and ergonovine in DHG. It is clear that some alkaloids show a definite accumulation with increased mowing height in both tall fescue and perennial ryegrass [22]. Our research with DHG demonstrates a similar effect on the accumulation of ergot alkaloids.

It appears possible to influence the levels of endophytic alkaloids in DHG via mowing (e.g., frequency and height). Li *et al*. [13] demonstrated that endophytes can improve host resistance to the insect pests *Rhopalosiphum padi* and *Tetranychus cinnabarinus*. This type of resistance also occurs in many other turf grasses, and, as a consequence, some insect pests might be controlled through this mechanism [17–20]. Therefore, alkaloid enhancement in DHG could have important implications for pest management when this species is used as a turf grass.

However, DHG has excellent forage potential if alkaloid production can be inhibited or reduced, as its protein content can reach 15% [33]. Even with alkaloids present, livestock will graze this grass if winter forage is insufficient. Sward height is an important factor for animal grazing. Sheep, for example, exhibit a more consistent rate of grass intake when the tiller length is 7.7 cm (7.1 g dry matter min^{-1}) than when it is 3.7 cm (1.0 g dry matter min^{-1}) [34]. Mowing this grass at a greater height also facilitates harvesting and storage. However, the present study demonstrates that DHG alkaloids are likely to increase under this treatment. This remains one of the main challenges in establishing this grass species as a significant forage crop.

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