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TEMPORAL AND SPATIAL VARIATION IN ALKALOID LEVELS IN *Achnatherum robustum*, A NATIVE GRASS INFECTED WITH THE ENDOPHYTE *Neotyphodium*

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Abstract—The native North American perennial grass *Achnatherum robustum* (Vasey) Barkworth [= *Stipa robusta* (Vasey) Scribn.] or sleepygrass is toxic and narcotic to livestock. The causative agents are alkaloidal mycotoxins produced from infections by a systemic and asexual *Neotyphodium* endophyte. Recent studies suggest that toxicity is limited across the range of sleepygrass in the Southwest USA. We sampled 17 populations of sleepygrass with varying distance from one focal population known for its high toxicity levels near Cloudcroft, NM, USA. For some, we sampled individual plants twice within the same growing season and over successive years (2001–2004). We also determined infection levels in each population. In general, all populations were highly infected, but infection levels were more variable near the focal population. Only infected plants within populations near the Cloudcroft area produced alkaloids. The ergot alkaloid, ergonovine, comprised the bulk of the alkaloids, with lesser amounts of lysergic and isolysergic acid amides and ergonovinine alkaloids. Levels of all alkaloids were positively correlated among individual plants within and between growing seasons. Infected plants that produced no alkaloids in 1 yr did not produce any alkaloids within the same growing season or in other years. Levels of alkaloids in sleepygrass populations declined with distance from the Cloudcroft population, although infection levels increased. Infected plants in populations in northern New Mexico and southern Colorado produced no alkaloids at all despite 100% infectivity. Our results suggest that only specific

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Neotyphodium haplotypes or specific *Neotyphodium*–grass combinations produce ergot alkaloids in sleepygrass. The *Neotyphodium* haplotype or host–endophyte combination that produces toxic levels of alkaloids appears restricted to one locality across the range of sleepygrass. Because of the wide variation in alkaloid levels among populations, interactions between the endophyte and host, and consequences for herbivores, competitors, and pathogens and other components of the community, are likely to vary widely across the geographic range of this native grass.

Key Words—*Achnatherum robustum*, alkaloids, endophyte, ergonovine, geographical variation, lysergic acid amide, native grass, *Neotyphodium*, sleepygrass.

INTRODUCTION

Asexual, vertically transmitted endophytes in the genus *Neotyphodium* are renowned for causing livestock toxicosis and reduced invertebrate herbivory in two introduced and widespread agronomic grasses, tall fescue (*Lolium arundinaceum*) and perennial ryegrass (*Lolium perenne*) (Clay, 1988, 1990; Siegel and Bush, 1996; Schardl and Phillips, 1997; Clay and Schardl, 2002). The toxic and deterrent properties of these *Neotyphodium*-infected agronomic grasses to vertebrate and invertebrate herbivores result from alkaloids produced by the endophytes. Increased resistance to herbivores and seed predators via endophytic alkaloids is generally viewed as the primary route of mutualistic benefits provided by *Neotyphodium* to the host grass (Cheplick and Clay, 1988; Saikkonen et al., 1998; Clay and Schardl, 2002), although *Neotyphodium* infections may also increase resistance to drought (Bacon, 1993) or poor soil nutrient conditions (Malinowski and Belesky, 1999), and generally increase competitive abilities (Marks et al., 1991; Clay et al., 1993). However, because alkaloids are nitrogen-rich compounds, benefits gained via protection against herbivores may be offset by reduced nitrogen availability to other host grass growth and reproductive functions, similar to other defensive allelochemicals produced by the host plant (Faeth, 2002).

The types and levels of alkaloids in agronomic grasses vary with host and especially with endophyte genotype (Siegel et al., 1990; Bush et al., 1993; Christensen et al., 1993; Leuchtmann et al., 2000; Bony et al., 2001). Recently, genes for alkaloid production in some *Neotyphodium* endophytes have been identified and manipulated (Spiering et al., 2005). Alkaloid levels in agronomic grasses are also influenced by environmental factors, such as soil moisture and nitrogen availability (Arechavaleta et al., 1992; Agee and Hill, 1994; Roylance et al., 1994; Malinowski et al., 1998; Hunt et al., 2005) and temperature (Huizing et al., 1991). Additionally, alkaloid production in tall fescue can be induced to higher levels by herbivory (Bultman et al., 2004).

Less is known about infection frequencies and alkaloid levels and types in native grasses, and particularly how alkaloids vary geographically or temporally. Some native grasses have long been known anecdotally as toxic to livestock (Hance, 1876; Bailey, 1903; Freeman, 1904; Marsh and Clausen 1929), but only recently has this toxicity been directly linked to infection by *Neotyphodium* endophytes (Kaiser et al., 1996; Miles et al., 1996; Jones et al., 2000; Moon et al., 2002). Early reports implied that infected grasses were toxic throughout their geographic range, and hence, common names such as "sleepygrass'' (*A. robustum*) and "drunken horse grass'' (*Achnatherum inebrians*) were ascribed to these species. However, recent studies suggest that many, if not all, of "toxic'' grass species with *Neotyphodium* infections are toxic in only some parts of their range (Miles et al., 1996; Jones et al., 2000; Faeth, 2002). Limited toxicity in *Neotyphodium*-infected grasses also seems to hold for the widely planted agronomic grasses, perennial ryegrass (Bony et al., 2001), and tall fescue (Saikkonen, 2000; Saikkonen et al., 2004), when examined in their native ranges. This suggests that alkaloid types and levels vary widely within and among populations of *Neotyphodium*-infected native grasses known for high toxicity. Alkaloid concentrations may also vary within and among growing seasons with environmental (e.g., soil moisture and nutrients) and developmental (e.g., as endophytes grow with their hosts) factors (Belesky and Hill, 1997), adding additional variation to alkaloid levels in native grass populations.

To our knowledge, geographic and temporal alkaloid variation in native *Neotyphodium*-infected grasses have yet to be simultaneously documented for any of the known highly toxic grasses (about 9–10 species; Faeth, 2002). Yet, alkaloid variation is the key trait influencing herbivore, seed predator and pathogen resistance, community interactions, and ecosystem functions (Matthews and Clay, 2001; Omacini et al., 2001; Faeth, 2002; Rudgers et al., 2004; Müller and Kraus, 2005). In previous studies, samples from individual plants have been pooled from within or across populations (Jones et al., 2000), individual infected plants have not been sampled over time (Powell and Petroski, 1992), or sampled plants were grown from seeds collected from natural populations (Miles et al., 1996). To assess within and among population variation, as well growing season variation, it is necessary to: (1) sample individual plants from multiple populations and (2) sample the same individual plants repeatedly over time. This populational variation is essential to understanding the coevolution and ecology of species interactions in general (Thompson, 1994, 2005) and, specifically, the outcome of interactions between host grass and systemic endophytes (Faeth and Sullivan, 2003; Sullivan and Faeth, 2004).

To ascertain geographic and temporal variation in alkaloids, we sampled 17 populations of sleepygrass or robust needlegrass, *A. robustum* [Vasey]

Barkworth = *Stipa robusta* [Vasey] Scribn.], radiating outward from the known toxic population in the Sleepygrass Picnic area, Lincoln National Forest, NM, USA, near the town of Cloudcroft (Petroski et al., 1992), from 2001 to 2004. We assessed the frequency of *Neotyphodium* infections in all populations. In some populations, individual plants were marked and resampled both within (seasonal) and among growing seasons (yearly). We correlated within and between growing season alkaloid levels to estimate how much variation is caused by developmental or environmental factors. We also determined how alkaloid levels varied with distance from the Sleepygrass Picnic area site, known for toxicity to livestock.

METHODS AND MATERIALS

Study Grass and Endophyte. A. robustum (Vasey) (Pooideae: Tribe Stipeae) is a cool-season, native grass found at high elevations throughout Arizona, New Mexico, Colorado, Wyoming, and Montana on "sky islands" of the Rocky Mountains in the western USA (Jones et al., 2000). Sleepygrass is a perennial bunchgrass found in semiarid pine–grassland habitats and reproduces by seed (USDA, 1988). In some localities in New Mexico, it is referred to as "robust needlegrass" (Jones et al., 2000).

The *Neotyphodium* endophyte is commonly found in sleepygrass populations at high frequencies (Petroski et al., 1992; Jones et al., 2000). *Neotyphodium* in *A. robustum* has yet to be identified to species (Moon et al., 2004; Schardl, personal communication). Morphologically, it appears intermediate in characteristics between *Neotyphodium starii* and *N. chisosum* (Kaiser et al., 1996) and also different from *Neotyphodium* isolated from *A. inebrians*, an Asian grass species known to produce high levels of similar alkaloids (Miles et al., 1996). Moon et al. (2004) found that *Neotyphodium* in sleepygrass was genetically most related to the sexual forms *Epichloë festucae* and *E. elymi*. Here, we refer to the endophyte as simply *Neotyphodium*.

Populations and Sampling. From 2001 to 2004, we sampled 17 populations of sleepygrass in New Mexico and Colorado (Table 1). We centered our sampling on a population that has long been known for high toxicity (Marsh and Clawson, 1929; Smalley and Crookshank, 1976; Jones et al. 2000), Sleepygrass Picnic area in the Lincoln National Forest (SP, Table 1), and then we sampled populations outward from this locality. The SP site is at the southern edge of the Sacramento Mountains, and most populations of sleepygrass occur northward. Three populations (SC, GR, and WD, Table 1), however, were southward, but still occurred within the Sacramento Mountain range. We assumed that these sites were separate populations, given that the closest population (MV) was 2.5 km from SP. *Neotyphodium* in Arizona fescue exhibits low gene flow between

populations (much less than 2.5 km) because seeds in which it is transmitted are not dispersed far (<1 m) and fall near the maternal plant (Sullivan and Faeth, 2004). Seeds of *A. robustum* may be transported further because of relatively large awns that may attach to animal fur, but we expect little movement between these disparate populations.

Number of sampled plants varied among years at the different sites (Table 1) because of time constraints, accessibility, missing plants from year to year, and replacement plants added in successive seasons or years. We sampled 432 plants among the 17 populations. In each population at each sampling period, leaf sheath tissue was removed, and plants were marked with surveyor's whiskers and numbered metal tags. Plant tissue was placed on ice and returned to the lab for determination of infection status and for alkaloid analyses. For 4 populations, the same plants were sampled twice within the same growing season, June and September 2003, at the beginning and the end of the growing season, respectively.

Infection and Alkaloid Analyses. Infection status of all plants was determined by a modified tissue immunoblot assay (Gwinn et al., 1991; Schulthess and Faeth, 1998) and was later confirmed by staining and microscopic examination of seeds for characteristic *Neotyphodium* hyphae (Saha et al., 1988). The remainder of all plant samples were frozen until they could be lyophilized. Freeze-dried samples were ground to a fine powder in a Wiley mill. Ergot alkaloids were analyzed by methods adapted from TePaske et al. (1993) and Jones et al. (2000). In brief, 5 ml methanol and 0.050 ml of concentrated ammonium hydroxide were added to 100 mg of ground plant tissue. Samples were rotated overnight and filtered. The filtrate was dried at 60°C under a flow of nitrogen, after which 2 ml of 1% acetic acid and 2 ml of chloroform were added, and the samples were mixed by rotation for 2–3 min. Samples were centrifuged to aid in layer separation, and a 1.0-ml aliquot of the upper acid solution was removed and placed into an high-performance liquid chromatography (HPLC) autosampler vial. Concentrated ammonium hydroxide was added (~0.030 ml) to adjust the pH to 9–10 along with 0.010 ml of a 100-ppm stock solution of monocrotaline (quantitative reference standard). Samples were analyzed by HPLC–mass spectrometry (MS). Separation and detection were achieved with a Betasil C18 reversed-phase HPLC column (100 × 2 mm, Keystone Scientific) and HP 1100 binary HPLC solvent pump coupled to an LCQ (Thermo Finnigan) mass spectrometer. Solvent elution was a gradient of methanol (A) and 20 mM ammonium acetate (B) starting at 25% (A) increasing to 55% (A) from 0 to 10 min. The mass spectrometer was operated under positive electrospray ionization and single ion monitoring of m/z = 268.3 and 326.3. Alkaloids are reported as total alkaloids as a mixture of ergonovine, lysergic acid amide, isolysergic acid amide, and ergonovinine. Quantification of individual alkaloids was made by measurement of peak area vs. calibration

TABLE 1. STUDY SITE DESCRIPTIONS, LOCATION, GPS COORDINATES, ELEVATION, AND SAMPLE SIZES (*N*) FROM FALL (SEPTEMBER) 2001 TO FALL 2004

Site	Description	City, county, state	GPS	Elevation (m)	Sampling years (*N*)
SC	Black Bear Campground, Lincoln National Forest	Near Cloudcroft, Otero Co., NM	32° 56.459′N, 105° 44. 474′W	2703	F 2001 (16), F 2002 (17) S 2003 (15), F 2003 (17)
GR	Carrie Green Ranch	Near Cloudcroft, Otero Co., NM	32° 55.170′N, 105° 44.379′W	2642	F 2001 (20), F 2002 (14) F 2003 (12)
MS1	Hwy 244 right of way near Mescalero Apache Reservation	Near Mescalero, Otero Co., NM	33° 01.674′N, 105° 36.408′W	2285	F 2001 (10)
SP	Sleepy Grass Picnic Area, Lincoln National Forest	Near Cloudcroft, Otero Co., NM	32° 57.452′N, 105° 43.092′W	2597	F 2001 (12), F 2002 (22) S 2003 (30), F 2003 (28)
WD	Lincoln National Forest	Near Weed, NM. Otero Co., NM	32° 47.691′N, 105° 35.659′W	2262	F 2001 (12), S 2003 (14), F 2003 (24)

MS2	Hwy 244 right of way near Mescalero Apache Reservation	Near Mescalero, Otero Co., NM	33° 10.740′N, 105° 41.620′W	2312	F 2001 (12), S 2003 (11)
MV	Lincoln National Forest	Near Cloudcroft, Otero Co., NM	32° 56.505′N, 105° 44.275′W	2659	S 2003 (41), F 2002 (39) F 2003 (32)
WR	Walker Ranch, 16 Springs Road	Near Mayhill, Otero Co., NM	33° 25.181′N, 111° 48.147′W	2285	F 2003 (43)
A	Intersection of US 285 and Rd G6	Near Antonito, Conejos Co., CO	37° 05.400′N, 106° 00.584′W	2385	F 2004 (20)
B	Near intersection of US 285/RD 12S	Near Alamosa, Alamosa Co., CO	37° 24.059′N, 105° 54.357′W	2260	F 2004 (20)
C	64/84 Hwy south of I-17	Near Chama, Rio Arriba Co., NM	36° 51.437′N, 106° 34.599′W	2334	F 2004 (20)
D	Hwy 159, South of Hwy 160/159 junction	Near San Luis, Costilla Co., CO	N: 37° 16.713′ W: 105° 25.981′W 981′	2414	F 2004 (20)
E	San Isabel National Forest, Hwy 12	Near North Lake, Las Animas Co., CO	37° 14.636′N, 105° 02.302′W	2537	F 2004 (20)
G	Hwy 69, between mm 40–41	Near Westcliffe, Custer Co., CO	37° 54.491′N, 105° 19.770′W	2476	F 2004 (20)
J	US 50 between mm 214–215	Near Salida, Chaffee Co., CO	38° 31.303′N, 106° 05.861′W	2273	F 2004 (20)
K	US 50 between mm 159–160	Near Gunnison, Gunnison Co., CO	38° 32.667′N, 106° 52.743′W	2272	F 2004 (20)
L	Hwy 62, Between mm 5–6	Near Placerville, San Miguel Co., CO	38° 05.042′N, 108° 00.477′W	2323	F 2004 (20)

Samples were collected from some populations in June at the beginning of the growing season in 2003 (S 2003).
GPS = Global Positioning System.

curve generated using ergonovine standards. We assayed only for ergot alkaloids, the main alkaloid type found in *A. robustum* (Petroski et al., 1992; Jones et al., 2000); it is possible that other minor alkaloids were present but not detected by our methods.

Statistical Analyses. We summed all unique infected and uninfected plants (N = 432) within each population across seasons and years to determine infection frequencies (that is, no plant was included more than once for determining infection frequency). We determined yearly changes in alkaloids by correlating total levels and individual alkaloids in the same individual plants from 2001 to 2002 and from 2002 to 2003. If plants produced relatively the same levels from year to year, then we expect a strong positive correlation. Likewise, we correlated levels of alkaloids in the same individual plants in June 2003 (beginning of the growing season) to levels in September 2003 (end of the growing season) to ascertain if seasonal levels of alkaloids vary within a growing season.

To determine if alkaloids vary with distance among populations from the SP population, known for its high toxicity, we regressed mean total alkaloids and individual alkaloid fractions from each population with distance (in km) from this focal population.

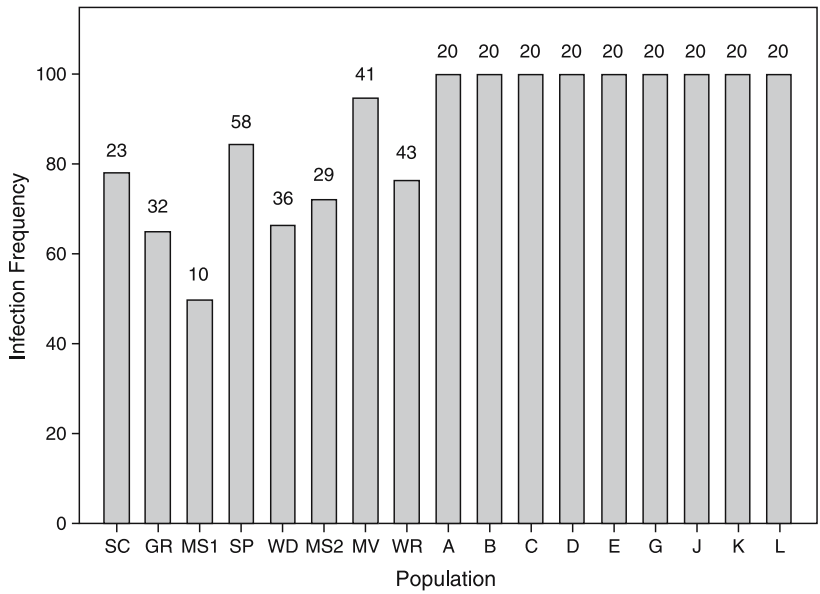

FIG. 1. Infection frequencies and sample sizes (numbers above bars) for the 17 sleepygrass populations. See Table 1 for site abbreviations and locations.

RESULTS

Infection Frequencies. Infection frequencies among populations were generally high, ranging from 50 to 100% (Figure 1). Notably, the northern New Mexico and southern Colorado populations (A–L, Figure 1) were 100% infected. Populations nearer the focal population SP were much more variable.

Alkaloid Levels. Total alkaloid levels were highly variable across populations (Figure 2). The highest levels were in infected plants in the focal SP population and the nearby MV and MS2 population (Figure 2). Some of the infected plants had >150 μg/g total ergot alkaloids, some of the highest known for *Neotyphodium*-infected plants. However, a few plants in these populations, although infected, never produced alkaloids, notably, the northern New Mexico and southern Colorado populations that were 100% infected (Figure 1, data not shown). Ergonovine was consistently the highest fraction with lesser amounts of lysergic and isolysergic amides (Figure 2). Ergonovinine constituted the lowest fraction (data not shown).

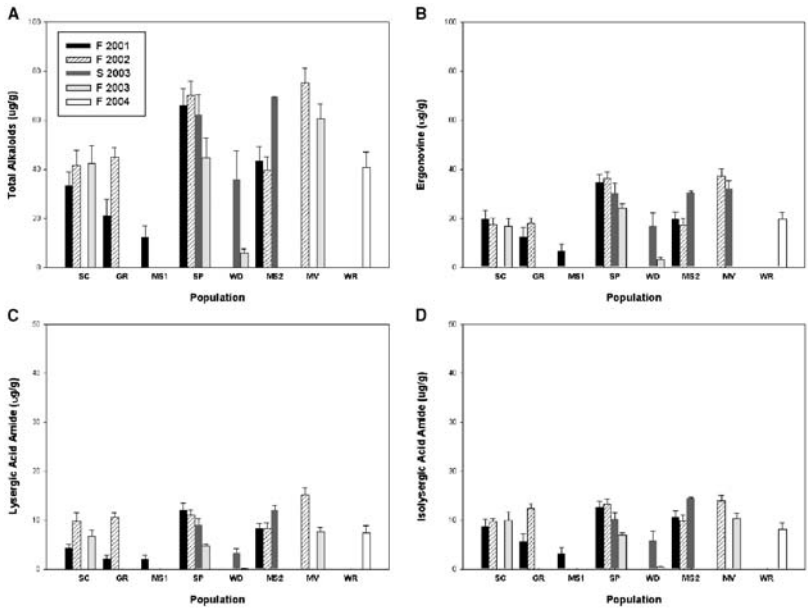

FIG. 2. Mean (±SE) concentrations (μg/g) of total (A), ergonovine (B), lysergic acid amide (C), and isolysergic amide (D) alkaloids from eight populations from 2001 to 2004. Infected plants from nine other populations in northern New Mexico and southern Colorado had zero alkaloids and are not shown. See Table 1 for site abbreviations. Note the difference in scale in boxes A, B and C, D.

Alkaloid levels in infected plants were positively correlated from 1 yr to the next. For the same individual plants sampled in fall of 2001 and 2002 (N = 16), concentrations of total (Pearson's correlation coefficient = 0.56, Bonferroni probability = 0.02), ergonovine (0.62, P = 0.01), isolysergic acid amide (0.53, P = 0.04), and ergonovinine (0.54, P = 0.03) were positively correlated. Levels of lysergic acid amides were not significantly correlated (P = 0.26). For the same individual plants sampled in fall of 2002 and 2003 (N = 50), concentrations of total (0.33, P = 0.02), ergonovine (0.32, P = 0.03), lysergic acid amides (0.42, P = 0.002), and isolysergic acid amide (0.38, P = 0.007) were positively and significantly correlated. Levels of ergonovinine alkaloids in fall of 2002 and 2003 were positively correlated (0.27), but only marginally significantly so (P = 0.06).

Within a growing season, alkaloid concentrations of the same individual plants (N = 20) were strongly and positively correlated. Levels of total alkaloids (0.71, $P < 0.001$), ergonovine (0.75, $P < 0.001$), lysergic acid amides (0.70, P = 0.001), isolysergic acid amides (0.64, P = 0.002), and ergonovinine (0.69, P = 0.001) were positively and significantly correlated in the same plants sampled in the beginning (June 2003) and end (September 2003) of the growing season.

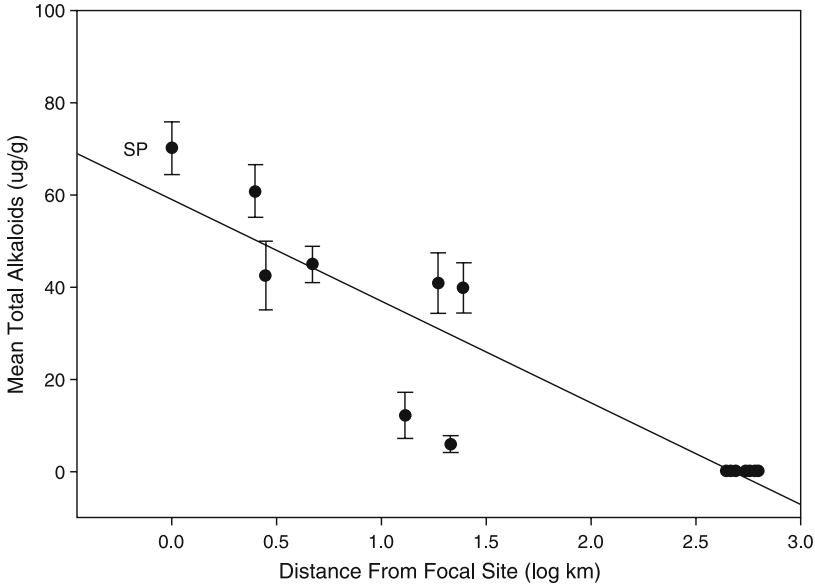

FIG. 3. Regression of mean (±SE) total alkaloids and log distance from site SP. Distances (km) were measured from the SP, the population used as the focal site (see text).

Mean Alkaloid Levels and Distance From The Focal Population. Mean levels of alkaloids in infected plants within each sampled population declined with linear distance from the focal population (SP; Figure 3). The best regression fit was alkaloid levels with log distance. Regressions of alkaloids levels with log distance were significant for mean total alkaloids ($N = 17$, adjusted $R^2 = 0.83$, $P < 0.001$, Figure 3), ergonovine (adjusted $R^2 = 0.80$, $P < 0.001$, data not shown), lysergic acid amides (adjusted $R^2 = 0.74$, $P < 0.001$, data not shown), isolysergic acid amides (adjusted $R^2 = 0.74$, $P < 0.001$, data not shown), and ergonovinine (adjusted $R^2 = 0.76$, $P < 0.001$, data not shown). Because all infected plants in the northern New Mexico and southern Colorado populations had no alkaloids, inclusion of all eight data points (Figure 3) could disproportionately skew the regression of alkaloid levels and distance towards a more negative relationship. Therefore, we also performed similar regressions of distance and alkaloid levels, but considered these eight distant populations as a single data point. The results were the same; mean total alkaloids and all mean alkaloid fractions rapidly and significantly declined with distance from the SP population.

DISCUSSION

Systemic fungal endophytes in cool-season, pooid grasses are well known for producing a wide variety of alkaloids that purportedly provide protection for the host against vertebrate and invertebrate herbivores (Cheplick and Clay, 1988; Breen, 1994; Siegel and Bush, 1996; Clay and Schardl, 2002; Faeth, 2002), root nematodes (West et al., 1988; Kimmons et al., 1990), some microbial pathogens (Burpee and Bouton, 1993), and possibly act as allelopathic agents (Peters and Zam, 1981). These effects are well known in two widespread agronomic grasses, tall fescue and perennial ryegrass, where *Neotyphodium* alkaloids are directly linked to livestock toxicoses and neurological disorders and increase resistance to generalized invertebrate pests (Bush et al., 1997; Schardl and Phillips, 1997). Much less is known about alkaloid levels in native grass populations harboring *Neotyphodium*. In general, relatively few *Neotyphodium*-infected native grasses are known for toxicity to livestock (Faeth, 2002). However, one of these, *A. robustum*, is toxic to horses, cattle, and sheep (Bailey, 1903; Marsh and Clawson, 1929; Petroski et al., 1992). Despite the central role of fungal alkaloids in agronomic and livestock production (Schardl and Phillips, 1997), and more generally to *Neotyphodium*–host grass interactions (Clay and Schardl, 2002; Faeth, 2002), there have been no studies of seasonal and yearly variation in alkaloid levels, or the relationship of alkaloid production to infection levels among populations, in infected native grasses.

Here, we show that ergot alkaloid levels in native sleepygrass populations are highly variable both within and among populations. The populations near Cloudcroft, NM, have high levels of ergot alkaloids on average, as previously reported (Petroski et al., 1992; Jones et al., 2000), but levels vary greatly, with some infected plants producing no alkaloids at all, whereas others produce more than 150 μg/g total alkaloids (range = 0–156 μg/g). Most of the alkaloid fraction is ergonovine, with lesser amounts of lysergic acid, isolysergic amide, and ergonovinine. This is in contrast to reports from Petroski et al. (1992) where ergonovine was lower relative to lysergic and isolysergic amides and other alkaloids. However, those plants were not sampled over time. In a study of the alkaloid content of a congeneric infected native grass from Asia, Miles et al. (1996) found that infected *A. inebrians* had high levels of ergonovine (up to 2500 μg/g) and lesser amounts of lysergic acid amide (up to 400 μg/g), similar to the relative amounts of alkaloids in sleepygrass but higher in absolute concentrations. Those plants, however, were grown in the greenhouse from seed, so it is not clear if the levels reflect alkaloid levels in natural populations. Nonetheless, in terms of alkaloid profiles, *Neotyphodium* in *A. robustum* and *A. inebrians* appear similar. Molecular studies would be fruitful to determine if *Neotyphodium* in these two geographically disjunct grass species are genetically related.

Lysergic and isolysergic acid amides are likely the alkaloids responsible for the dramatic narcotic effects of infected sleepygrass on livestock (Miles et al, 1996; Jones et al., 2000). Horses that feed on small amounts of infected sleepygrass tissue or seeds from the Cloudcroft region become narcotized and go into a deep sleep (Powell and Petroski, 1992). Consumption of larger amounts causes elevated body temperature, weakness, frequent urination, dizziness, hypersalivation, diarrhea, and possibly death (Smalley and Crookshank, 1976; Miles et al., 1996). Both lysergic acid and ergonovine are vasoconstrictors. Ergonovine is known as a stimulator of smooth muscles and used medically to induce uterine contractions (Powell and Petroski, 1992; Miles et al., 1996). Ergot alkaloids, in general, are deterrent and toxic to some invertebrate herbivores (Siegel et al., 1990), but it is not known if ergonovine generally deters herbivory by vertebrates or invertebrates as do lysergic acid amides, although vasoconstriction is associated with heat stress and tissue gangrene in livestock consuming other *Neotyphodium*-infected grasses. Ergonovine may also increase susceptibility of invertebrate herbivores to entomopathogenic nematodes (Kunkel et al., 2004).

That alkaloid levels are positively correlated both within and among growing seasons suggests that the endophyte species, endophyte haplotype, or the host–endophyte genotypic combination largely determines alkaloid levels. Although alkaloids varied slightly within population within and among growing seasons, in general, levels remained consistent (Figure 2), indicating that

environmental and developmental variation has lesser influences on alkaloid levels in infected sleepygrass. Alkaloid variation in infected agronomic grasses and some other native grasses appears largely dictated by endophyte species or haplotypes (Siegel et al., 1990; Leuchtmann, 1992, 1997; Bush et al., 1993; Christensen et al., 1993) and less so by host genotype and environmental factors (Siegel et al., 1990; Leuchtmann et al., 2000). However, host grass genotype in agronomic grasses (Siegel et al., 1990) and at least one infected native grass may drive alkaloid production (Faeth et al., 2002). Although we cannot distinguish here between endophyte and host genotype effects, or their interactions, on alkaloid production, consistent levels within and between growing seasons suggest that environmental factors play a lesser role. Furthermore, infected plants that produced no alkaloids in June never did so in the fall, and infected plants that produced no alkaloids in 1 yr did not in subsequent years, suggesting that a specific *Neotyphodium* species or haplotypes or specific host and endophyte genotypic combinations are necessary for alkaloid production.

That alkaloid levels in sleepygrass populations decline and disappear with distance from the SP population suggests that the specific strain or haplotype of *Neotyphodium* or endophyte–host genetic combination may be localized near Cloudcroft, NM. This strain or combination rapidly decreases with distance in frequency relative to less toxic and nontoxic strains or combination. The *Neotyphodium* endophyte in sleepygrass has yet to be identified to species or thoroughly examined for geographic genetic variation. Kaiser et al. (1996) found a diversity of spore morphologies in cultures of 10 New Mexico and Colorado populations. Moon et al. (2004) found a hybrid *Neotyphodium* in sleepygrass in one sample from southern Colorado. Based on gene sequences, they postulated that this hybrid was formed from *Epichloë elymi* and *E. festucae*. Hybridization is thought to occur when the sexual and spore-transmitted form *Epichloë* colonizes and hybridizes with *Neotyphodium* in the same host grass or when two *Epichloë* species hybridize. Hybridization is thought to rapidly infuse genetic variation into the population and create novel genetic combinations that may result in new alkaloid types (Schardl and Craven, 2003; Moon et al., 2004). The hybrid *Neotyphodium* in Moon et al. (2004) came from regions in southern Colorado where we found no alkaloids. Furthermore, the hybrid in Moon et al. (2004) does not harbor the gene for ergot alkaloids (Schardl, personal communication). In another native southwest US grass, Arizona fescue (*Festuca arizonica*), genetic variation in *Neotyphodium* is highly spatially structured, with the presence of hybrid and nonhybrid endophytes, and with little or no gene flow between grass populations that are less than 2 km apart (Sullivan and Faeth, 2004). Sullivan and Faeth (2004) also found that hybrids did not differ in levels or types of alkaloids relative to Arizona fescue with nonhybrid *Neotyphodium*. Thus, hybridization at least in native sleepygrass and Arizona fescue apparently is not a prerequisite for novel or high alkaloid

production. Future studies should focus on ascertaining the underlying genetic basis in the *Neotyphodium* endophyte for this steep gradient in alkaloid production in sleepygrass populations.

Interestingly, the sleepygrass populations near Cloudcroft are also more variable in infection frequency than northern New Mexico and southern Colorado populations (Figure 1). Infection frequency in the latter are 100%, but the *Neotyphodium*–grass symbiota produces no alkaloids. The variability in infection frequency and in alkaloid levels among infected plants near the SP population suggests that high alkaloid producing *Neotyphodium* endophytes may also incur substantial costs. Faeth (2002) argued that alkaloid production in infected grasses is costly in terms of nitrogen demand and metabolic costs. High levels may also be costly in terms of toxicity to the host, similar to alkaloidal allelochemicals produced directly by host plants (Karban and Baldwin, 1997). The benefits of high alkaloids may offset these costs when grazing or herbivory is intense and persistent and available soil nutrients are high (Faeth, 2002). The frequency of toxic infected *A. robustum* (Marsh and Clawson, 1929; Jones et al., 2000) and *A. inebrians* (Miles et al.,1996; Nan and Li, 2001) appears to have increased relative to nontoxic individuals because livestock grazing has intensified in the respective geographic range of these host grasses.

Our results indicate that *A. robustum*–endophyte interactions, in terms of alkaloids, are highly spatially structured across a wide geographic range of populations. Thompson's (1994, 2005) geographical mosaic theory predicts that interactions evolve or coevolve in metapopulations, with the interaction between local selection pressures, migration, and genetic drift creating a range of possible outcomes in species interactions geographically. Alkaloid levels in infected sleepygrass vary dramatically, and the notion of sleepygrass as a toxic grass because of endophyte infection only applies in a very restricted part of the geographic range of the host grass. Given that alkaloid production is considered the main trait that dictates the outcome of grasses infected with systemic, seed-borne endophytes with herbivores, pathogens, competing plants, higher trophic levels, community diversity, and even ecosystem functions (Saikkonen et al., 1998; Omacini et al., 2001; Clay and Holah, 1999; Clay and Schardl, 2002; Faeth, 2002; Faeth and Bultman, 2002; Rudgers et al., 2004), we predict that these interactions, and community and ecosystem repercussions, will also be highly variable across the geographic range of the sleepygrass. Understanding populational variation in alkaloids because of endophyte and host genetics, and not just infection status of grasses, will be necessary to unravel these complex host–endophyte interactions and their consequences at the community and ecosystem level.

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