Investigating the Allelopathic Potential of *Kalmia latifolia* L. (Ericaceae)

Holly R. Eppard[1], Jonathan L. Horton[1,3,*], Erik T. Nilsen[1], Preston Galusky[1], and Barton D. Clinton[2]

Abstract - Evergreen, understory shrubs, often members of the Ericaceae, have been implicated in the suppression of tree recruitment in many ecosystems. One possible mechanism of this suppression could be an allelopathic interaction between shrubs and seedlings. We tested the allelopathic potential of *Kalmia latifolia* L., an important component of southern Appalachian forest understories. Aqueous extracts of soil, humus, *K. latifolia* litter, mixed forest floor litter, and green leaves from *K. latifolia* did not inhibit germination or initial root elongation of our test species *Lactuca sativa* L. (lettuce). Root, shoot, and total biomass were reduced in *P. rigida* seedlings grown in forest floor substrate collected beneath *K. latifolia* compared to those grown in forest substrate without *K. latifolia*. While these differences were not significant, they could be exacerbated in the field when seedlings are exposed to multiple stressors (reduced light, water, and nutrient availability). Our findings suggest that allelopathy is not a strong mechanism in the inhibition of canopy tree recruitment by *K. latifolia*.

Introduction

Kalmia latifolia L. (Ericaceae) is an evergreen understory shrub found in abundance on xeric, southwest slopes in the southern Appalachian Mountains. *Kalmia latifolia* typically forms a dense understory in mixed pine/hardwood stands and occasionally in association with *Rhododendron maximum* L. (Clinton et al. 1994, Lipscomb and Nilsen 1990, Woods and Shanks 1959). The chestnut blight of the 1920s, as well as the lack of a major disturbance since, has facilitated the establishment of *K. latifolia* in the Southern Appalachians (Brose et al. 2002). According to Brose et al. (2002), the presence of *K. latifolia* as a prominent understory shrub has ultimately contributed to a reduction of *Pinus pungens* Lamb. (Table Mountain pine) and *Pinus rigida* Mill. (pitch pine) regeneration (Brose et al. 2002).

Ericaceous shrubs are implicated in the inhibition of canopy tree recruitment in many forested ecosystems including *Gaultheria shalon* Pursh. in coniferous forests of the Pacific Northwest (Klinka et al. 1989, Messier 1993), *Rhododendron ponticum* L. in the British Isles (Cross 1981, Fuller and Boorman 1977, Mitchell et al. 1997), *Rhododendron maximum* in the southeastern United States (Beier et al. 2004; Clinton et al. 1994; Lei et al. 2002; Nilsen et al. 1999, 2001), and *Kalmia*

[1]Biology Department, Virginia Tech, Blacksburg, VA 24061. [2]USDA Forest Service Southern Research Station, Coweeta Hydrologic Laboratory, Otto, NC 28763. [3]Current address - Department of Biology, University of North Carolina at Asheville, Asheville, NC 28804. *Corresponding author - jhorton@unca.edu.

angustifolia (Small) Ferald. in northeastern Canada (Mallik 1995). Furthermore, several studies have reported inhibition of canopy tree recruitment beneath *K. latifolia* in the southern Appalachians (Clinton et al. 1994, Monk et al. 1985, Waterman et al. 1995).

The dominance of a particular species in a community is often due to either a competitive advantage over the acquisition of resources, or the production of inhibitory compounds (Gant and Clebsch 1974). In addition to the obvious competitive nature that a dense understory will exhibit (e.g. reduced light availability), allelopathy may further inhibit seedlings beneath ericaceous shrubs. This mechanism is characterized by a release of chemical compounds from one plant which may directly or indirectly inhibit or stimulate another plant (Rice 1984). The release of chemicals can occur by various means, such as decomposition of leaf litter, foliar leaching, or root exudation. For example, Nilsen et al. (1999) found that litter leachates of *R. maximum* inhibited seed germination and root elongation of bioassay species, and reduced the growth of some ectomycorrhizal fungi (Nilsen et al. 1999). However, a direct allelopathic effect by *R. maximum* was not demonstrated in the field (Nilsen 2002). In addition, *K. angustifolia*, an understory shrub of the boreal forest, was shown to reduce *Picea mariana* Mill. (black spruce) regeneration via allelopathy (Mallik 1987, Peterson 1965). Furthermore, phenolic compounds isolated from aqueous extracts of *K. angustifolia* leaves were shown to be allelopathic to *P. mariana* (Zhu and Mallik 1994).

Given the allelopathic properties of closely related species to *K. latifolia*, we proposed the hypothesis that allelopathy was a potential mechanism for inhibition of canopy recruitment under *K. latifolia* in the southern Appalachian Mountains. To test this hypothesis we: 1) assessed the effect of aqueous leachates from various *K. latifolia* substrates on seed germination and root elongation of a fast-growing assay species, and 2) compared growth and survival of *Pinus rigida* seedlings grown in forest floor substrate (soil and humus) collected below *K. latifolia* thickets with those grown in forest floor substrate collected from areas where *K. latifolia* was absent.

Materials and Methods

Leachate preparation

Forest floor substrate and green *K. latifolia* leaves were collected several times in the spring and summer of 2002 (March–June) from sites containing a dense *K. latifolia* subcanopy at Coweeta Hydrologic Laboratory in the Nantahala Mountains of western North Carolina (35°02'N, 83°24'W; Swank and Crossley 1988). The collection sites consisted of xeric east-facing ridgetop locations (elevation 1171 m) that contained a mixed oak-hickory forest that is being used in a larger study on the inhibition of tree regeneration by understory evergreen shrubs. These sites were chosen so that results from this study could be integrated into the larger study. Forest substrate was separated into litter (distinguishable fallen leaves), humus (decomposing organic matter), and mineral soil. A subsample of the litter collection was

further separated into two groups, one containing *K. latifolia* leaf litter only and the other containing mixed litter from all forest species including that of *K. latifolia*. Samples of green leaves (close to abscission) were removed from the shrub. We prepared leachates for use in bioassays by placing substrate (*K. latifolia* green leaves, *K. latifolia* litter, mixed litter [forest floor with *K. latifolia*], humus, and mineral soil from under *K. latifolia*) in sealed bags with distilled water at a 1:5 substrate to water ratio by mass (Nilsen et al. 1999). These mixtures were incubated in a growth chamber for 24 h at 25 °C and gently-shaken periodically. Samples were filtered first through cheesecloth to remove large debris and again through Whatman Number 3 filter paper. Leachates were stored at 4 °C and used within 5 days after filtration. Leachate pH and osmolality were measured before all experiments using a pH meter (Fisher Accumet Model 610A; Fisher, Pittsburg, PA) and a vapor pressure osmometer (VAPRO model 5520; Wescor, Logan, UT), respectively.

Bioassay of solution toxicity

We used *Lactuca sativa* L. (lettuce: "Black-Seeded Simpson," all from same lot number) as a test species because of its rapid germination and frequent use in bioassay studies (Del Moral and Cates 1971, Rice 1979). To test the allelopathic effect of leachates on seed germination, we placed 30 seeds in a petri dish containing 2 layers of Whatman number 1 filters, which were moistened with 3 ml of leachates from green leaves, *K. latifolia* litter, mixed litter, humus, and mineral soil. Leachates were evaluated with distilled water serving as the control. The petri dishes were sealed with parafilm and placed in a growth chamber at 25 °C, 60% relative humidity, and a 12/12 h day/night (photosynthetic photon flux density 90 μmol m^{-2} s^{-1}). Germination percentage of each dish was calculated each day for five consecutive days after imbibing (maximum germination was reached by day five). The experiment was replicated seven times.

We tested the effect of leachates on root elongation by arranging 20 seeds in two rows down the middle of a petri dish containing 2 layers of Whatman number 1 filter papers. Each seed was oriented for root growth towards the edge of the plate. These petri dishes were moistened with 3 ml of leachate and incubated as above for five days. A calibrated scale was placed within each petri dish and the dish with germinating seeds was photographed (CCD Camera, model 4815-5100; Cohu, San Diego, CA) with a macro lens (50mm 1:3.5; Canon, Lake Success, NY) attached to a computer (Macintosh, model G4; Apple, San Jose, CA). Digital images were stored for later analysis. The scale provided in each dish was used to calibrate root length measurements made on the digital images using an image analysis program (NIH Image; Research Services Branch, National Institute of Health, http://rsb.info.nih.gov/nih-image). Root length of each germinated seed in all petri dishes was recorded each day for five consecutive days. Mean root length was calculated for each plate (treatment) on each sampling date. This experiment was replicated seven times.

Pinus rigida greenhouse experiment

In June 2002, we collected forest floor substrate (soil and humus) from five blocks with collections from sites with a dense *K. latifolia* subcanopy (Kl^+) and from five paired sites where *K. latifolia* was absent (Kl^-). Paired plots were within 10 m of each other. These five blocks were chosen within the forest at the Coweeta Hydrology Laboratory, where substrate collections for leachate preparations were made. Each block was more than 10 m away from each other. The soil/humus substrate was placed in 15-cm pots and transported to a greenhouse on the campus of Virginia Tech. Three cores from each site were collected for a total of 15 pots of each treatment (Kl^+ and Kl^-).

We obtained *Pinus rigida* seeds all of the same lot number from the USDA Forest Service, Erambert/Black Creek Seed Orchards. Seeds were placed under cold running water for 24 h, and 25 seeds per petri plate were imbibed with distilled water for three weeks between two saturated layers of Whatman number 1 filter paper in a growth chamber. Of the five hundred imbibed seeds, 37% germinated. We placed five germinated *P. rigida* seeds in each pot containing forest floor substrate. Seedlings were kept well watered with tap water and grown in the greenhouse for 15 weeks and survival for each pot was monitored weekly. Seedlings were harvested after 15 weeks and above and below ground biomass were measured after drying at 60 °C for 48 hours.

Data analysis

Differences in pH and osmolality of leachates and the distilled water control were assessed utilizing analysis of variance (ANOVA) with six different treatment levels (distilled water, green leaf, mixed litter, *K. latifolia* litter, humus, and soil). Differences in percent germination (arcsin-square root transformed) and root elongation were compared among the treatments each sampling day using repeated measures ANOVA. Differences in percent survival (arcsin-square root transformed) and mean aboveground, belowground and total biomass of pitch pine seedlings in each pot were compared between substrate types using one-way ANOVA with blocking by site (5 blocks with 3 replicates per block) for two treatment levels (total n = 30).

Results

Effects of aqueous extracts on lettuce germination and root elongation

There were no significant differences among treatments in pH or osmolality of leachates ($p > 0.05$; data not shown). Seed germination was reduced in each leachate compared to the distilled water control after one day ($F = 2.2$, $P = 0.076$; Fig. 1). Percent germination at day one was highest in the distilled water control, green leaf, and soil leachates, intermediate in the mixed litter and *K. latifolia* litter leachates, and was lowest in the humus leachate (Fig. 1). Germination in the humus leachate was consistently lower throughout the five day trial, although these differences were not significant (Fig. 1).

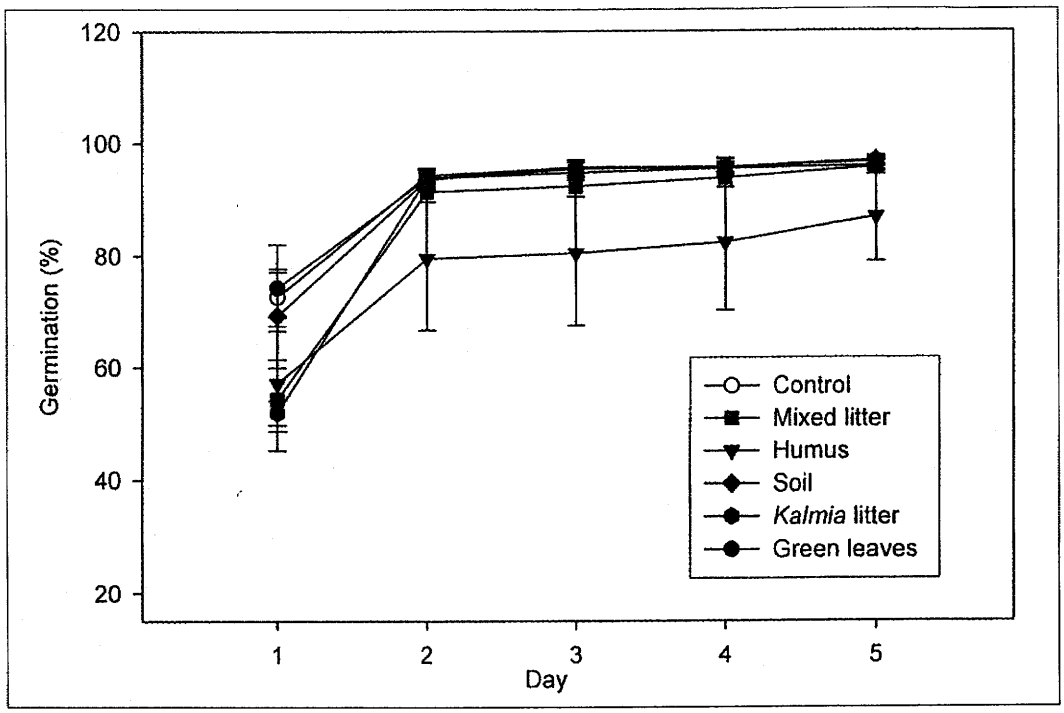

Figure 1. Mean (± 1 SE) seed germination in aqueous leachates (soil, humus, *K. latifolia* litter, mixed litter [including *K. latifolia*], *K. latifolia* green leaves) and a distilled water control. Seeds were imbibed on day zero and percent germination of each plate was determined each day for five days. Points represent the mean of seven replicates.

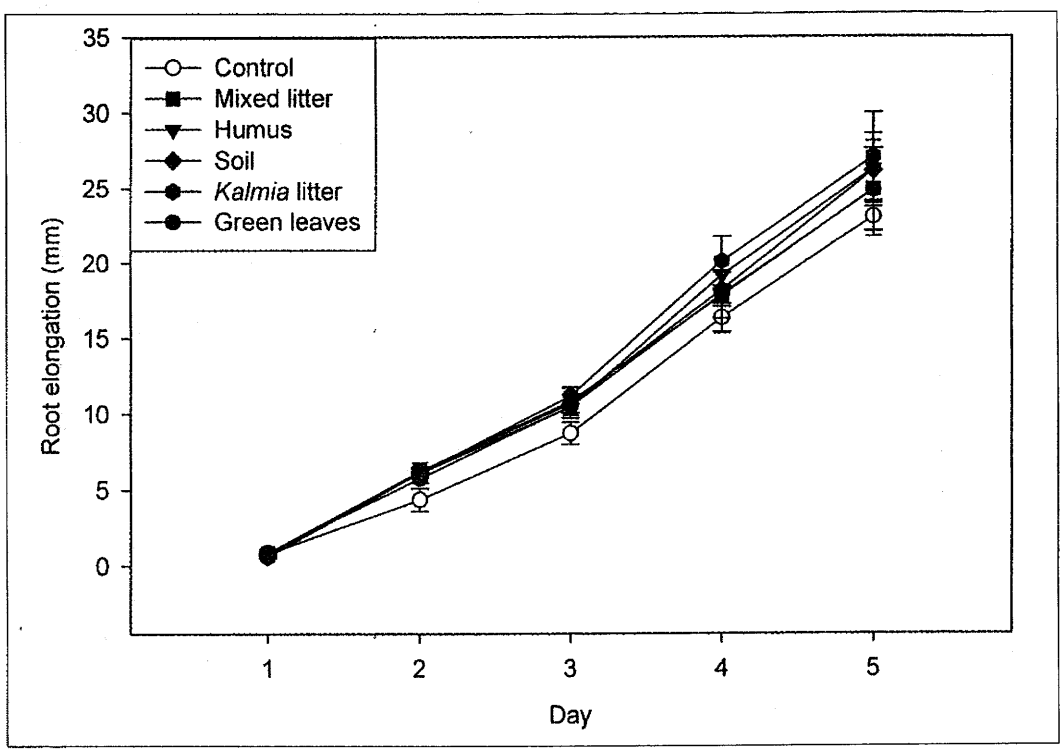

Figure 2. Mean (± 1 SE) root elongation in aqueous leachates (soil, humus, *K. latifolia* litter, mixed litter [including *K. latifolia*], *K. latifolia* green leaves) and a distilled water control. Seeds were imbibed on day zero and the mean root length of each germinated seed per plate was calculated each day for five days. Points represent the mean of seven replicates.

We found no significant differences in root elongation among any leachates or control over the course of the experiment (Fig. 2). Root elongation was consistently lower in the distilled water control than in the leachates. Although this difference increased throughout the course of the experiment, it was never significant.

Effect of *K. latifolia* substrate on *P. rigida* seedlings

Seedling survival after fifteen weeks was lower in the Kl^+ pots (42.8 ± 5.8%) than in the Kl^- pots (51.8 ± 5.0%), although these differences weren't significant (F = 0.61, P = 0.693). Both above (Fig. 3A) and belowground

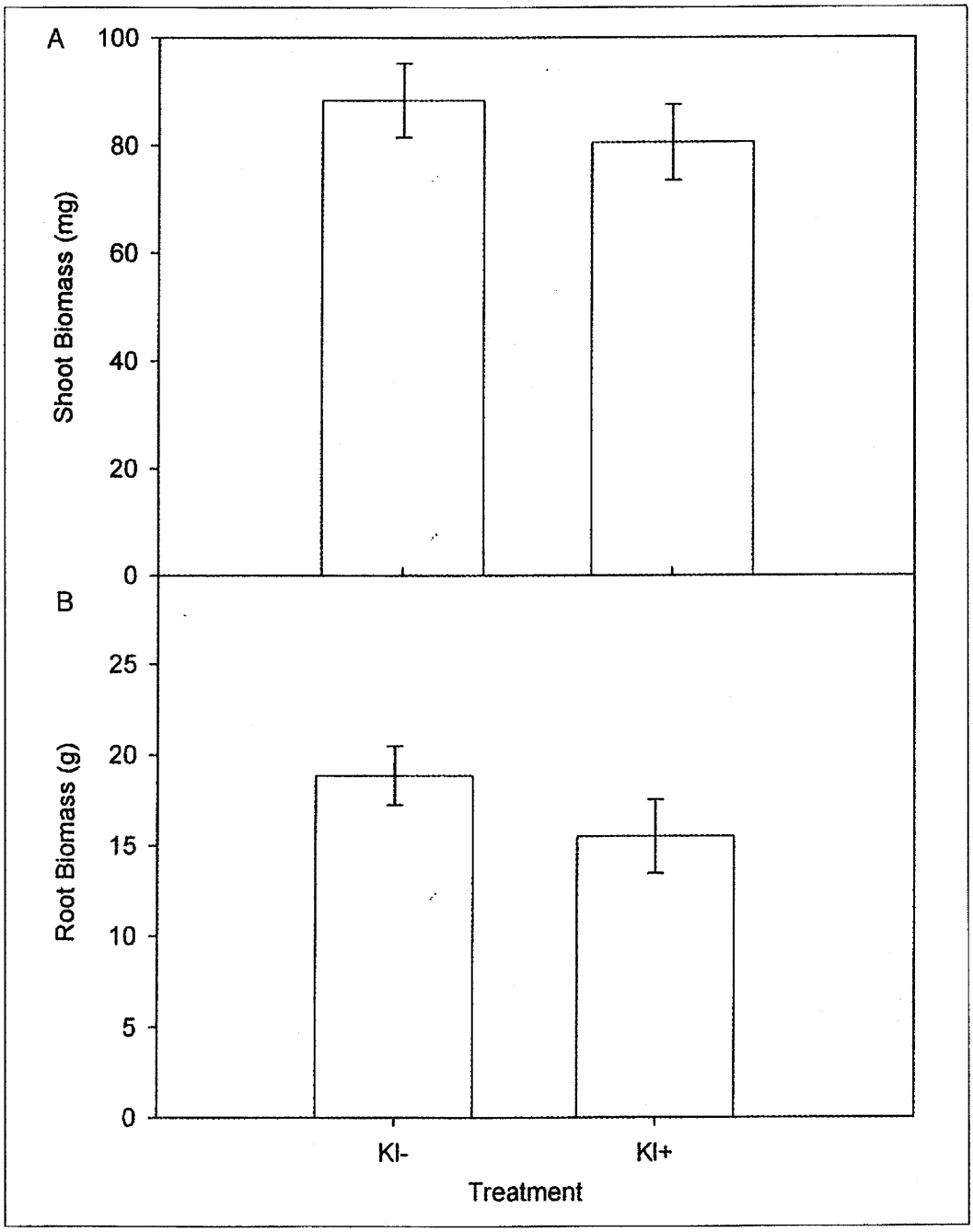

Figure 3. Mean (± 1 S.E.) shoot biomass (A) and root biomass (B) of harvested pitch pine seedlings grown in forest or *K. latifolia* substrate for 15 weeks.

(Fig. 3B) and total (Kl^- = 107.2 ± 12.3 mg; Kl^+ = 96.0 ± 12.6 mg) biomass of pitch pine seedlings grown on Kl^+ were reduced relative to those grown on Kl^- substrate; however, these differences were not significant (above: F = 0.38, P = 0.860; below: F = 1.94, P = 0.099; total: F = 0.67, P = 0.645).

Discussion

Kalmia latifolia has been associated with suppression of tree recruitment. One potential mechanism of this suppression is the production of allelopathic chemicals. Inderjit and Mallik (2002) propose three criteria that must be satisfied to demonstrate allelopathy. In the context of our study, they are: 1) Does *K. latifolia* possess toxic chemicals? 2) Do these organic compounds release and accumulate in the environment? and 3) Is allelopathy sufficient to explain *K. latifolia's* inhibition of tree seedlings? We will address each of these criteria separately.

Does *K. latifolia* possess toxic chemicals?

While we did not perform chemical analysis in this study, there is ample circumstantial evidence that *K. latifolia* contains toxic compounds. It has been noted that the foliage is toxic to livestock and possesses cytotoxic compounds (Mancini and Edwards 1979, Marsh and Clawson 1930). *Kalmia latifolia* is also evergreen and many related evergreen species have high phenolic content in their leaves as an anti-herbivore defense (Coley 1988). The closely related species, *K. angustifolia* has been shown to possess toxic phenolic compounds (Zhu and Mallik 1994), so it is probable that *K. latifolia* also possesses such phenolics.

Do these organic compounds release and accumulate in the environment?

We addressed this criterion in two ways, the first was to use aqueous extract solutions and the second was to use actual forest floor substrate collected from areas with and without *K. latifolia*. Inderjit and Dakshini (1995) and Inderjit and Nilsen (2003) advise caution in the design of allelopathic bioassays, recommending the use of unadulterated substrate in aqueous solutions to simulate natural conditions of chemical release. Many studies use macerated substrate, which may release chemicals that might not be naturally leached or exuded, and thus may not be ecologically relevant (Inderjit and Dakshini 1995). We followed the protocol described by Nilsen et al. (1999) in the formation of leachates for our bioassays. However, the substrate-to-water ratio that was used in this study may not simulate the concentrations of plant chemicals experienced in the field, but are likely more concentrated.

Our second experiment involving actual substrate from areas with and without *K. latifolia* was meant to better simulate field conditions. There were slight decreases in survival and both above- and belowground growth in those seedlings grown in the Kl^+ substrate, although these were not significant. It is possible that any allelochemicals that had accumulated in

the forest floor substrate leached out of the pots during the fifteen weeks of the study, and without living shrubs, there was no replacement of these chemicals via throughfall, litterfall, or root exudation. The pine seedlings grew very slowly and it is possible that the slight differences between treatments may have become more pronounced if the experiment were allowed to continue longer.

Is allelopathy sufficient to explain *K. latifolia*'s inhibition of tree seedlings?

Our results suggest that allelopathy is not a strong factor in the inhibition of seedling growth under *K. latifolia*. There was no significant effect on test species seed germination although, germination in the humus leachate was consistently lower and more variable than germination in the other leachates. This could indicate the accumulation of allelochemicals in the humus layer below *K. latifolia* and may affect seed germination under shrubs in the field. Root elongation was higher in all leachates than in the distilled water control. Although these differences were not significant they suggest a possible stimulation of elongation in the aqueous leachates relative to the control. This could be due to the fact that the distilled water control had no nutritional content to support root elongation and the *L. sativa* seeds had little endocarp to support elongation.

We found no differences in survival and small, but not significant, differences in biomass of *P. rigida* seedlings grown in substrate from under *K. latifolia* and substrate from forest sites without *K. latifolia*. The slight differences in *P. rigida* seedling biomass observed in this study could be exacerbated in the field when seedlings face multiple stressors (Rice 1984). There may be multiple stress factors induced by *K. latifolia* on tree seedlings including reduced light, water, and nutrient availability as seen with *R. maximum* in the southern Appalachians (Nilsen et al. 2001). Evergreen shrubs are known to alter soil carbon-to-nitrogen ratios, which could alter nutrient cycling processes and potentially reduce nitrogen availability (Inderjit and Mallik 1999). Additionally, ericaceous shrubs are shown to negatively affect mycorrhizal colonization (Walker et al. 1999, Yamasaki et al. 1998), which could further reduce seedling performance.

Kalmia latifolia is implicated in the suppression of tree regeneration in pine-hardwood forests in the southern Appalachian Mountains. This inhibition likely stems from a combination of factors including resource competition and potentially, allelopathy. Our bioassays have failed to determine a strong allelopathic potential in aqueous leachates of *K. latifolia* litter and humus. However, slight reductions in tree seedling performance due to the production of allelochemicals may be further exacerbated by a combination of multiple stressors resulting from competitive interactions between the shrubs and seedlings. The nature of these interactions should be addressed in more detail in field-based experiments to fully understand the inhibitory mechanisms involved.

Literature Cited

Beier, C.M., J.L. Horton, J.F. Walker, B.D. Clinton, and E.T. Nilsen. 2004. Resource availability and tree seedling performance along natural gradients of understory shrub density in an eastern deciduous forest. Plant Ecology 176:131–142.

Brose, P., F. Tainter, and T. Waldrop. 2002. Regeneration history of three Table Mountain pine/pitch pine stands in northern Georgia. General Technical Report SRS-48, US Forest Service, NC. 622 pp.

Clinton, B.D., L.R. Boring, and W.T. Swank. 1994. Regeneration patterns in canopy gaps of mixed-oak forests of the Southern Appalachians: Influences of topographic position and evergreen understory. American Midland Naturalist 132:308–319.

Coley, P.D. 1988. Effects of plant growth rate and leaf lifetime on the amount and type of anti-herbivore defense. Oecologia 74:531–536.

Cross, J.R. 1981. The establishment of *Rhododendron ponticum* L. in the Killarney oakwoods, S.W. Ireland. Journal of Ecology 69:807–824.

Del Moral, R., and R.G. Cates. 1971. Allelopathic potential of the dominant vegetation of western Washington. Ecology 52:1030–1037.

Fuller, R.M., and L.A. Boorman. 1977. The spread and development of *Rhododendron ponticum* L. on the dunes at Winterton, Norfolk, in comparison with invasion by *Hippophae rhamnoder* at Salt Fleetby, Lincolnshire. Biological Conservation 12:82–94.

Gant, R.E., and E.E.C. Clebsch. 1974. The allelopathic influences of *Sassafras albidum* in old-field succession in Tennessee. Ecology 56:604–615.

Inderjit, and K.M.M. Dakshini. 1995. On laboratory bioassays in allelopathy. Botanical Review 61:28–44.

Inderjit, and A.U. Mallik. 1999. Nutrient status of black spruce (*Picea mariana* (Mill.) BSP) forest soils dominated by *Kalmia angustifolia* L. Acta Oecologica 20:87–92.

Inderjit, and A.U. Mallik. 2002. Can *Kalmia angustifolia* interference to black spruce (*Picea mariana*) be explained by allelopathy? Forest Ecology and Management 160:75–84.

Inderjit, and E.T. Nilsen. 2003. Bioassays and field studies for allelopathy in terrestrial plants: Progress and problems. Critical Reviews in Plant Sciences 22:221–238.

Klinka, K., R.E. Carter, M.C. Feller, and Q. Wang. 1989. Relation between site index, salal, plant communities, and sites in coastal Douglas-fir ecosystems. Northwest Science 63:19–29.

Lei, T.T., S.W. Semones, J.F. Walker, B.D. Clinton, and E.T. Nilsen. 2002. Effects of *Rhododendron maximum* on tree seed dispersal, seedling morphology, and survivorship. International Journal of Plant Science 16:991–1000.

Lipscomb, M.V., and E.T. Nilsen. 1990. Environmental and physiological factors influencing the natural distribution of evergreen and deciduous ericaceous shrubs on northeast-and southwest-facing slopes of the Southern Appalachian Mountains. II. Water relations. American Journal of Botany 77:517–526.

Mallik, A.U. 1987. Allelopathic potential of *Kalmia angustifolia* to black spruce (*Picea mariana*). Forest Ecology and Management 20:43–51.

Mallik, A.U. 1995. Conversion of temperate forests into heaths: Role of ecosystem disturbance and ericaceous plants. Environmental Management 19:675–684.

Mancini, S.D., and J.M. Edwards. 1979. Cytotoxic principles from the sap of *Kalmia latifolia*. Journal of Natural Products 42:483–488.

Marsh, C.D., and A.B. Clawson. 1930. Mountain laurel (*Kalmia latifolia*) and sheep laurel (*Kalmia angustifolia*) as stock-poisoning plants. USDA Technical Bulletin # 219. US Department of Agriculture, Washington, DC. 23 pp.

Messier, C. 1993. Factors limiting early growth of western redcedar, western hemlock, and Sitka spruce seedlings on ericaceous-dominated clearcut site in coastal British Columbia. Forest Ecology Management 60:181–206.

Mitchell, R.J., R.H. Marrs, and R.H. LeDuc. 1997. A study of succession on lowland heaths in Dorset, south England: Changes in vegetation and soil chemical properties. Journal of Applied Ecology 34:1426–1444.

Monk, C.D., D.T. McGinty, and F.P. Day, Jr. 1985. The ecological importance of *Kalmia latifolia* and *Rhododendron maximum* in the deciduous forest of the southern Appalachians. Bulletin of the Torrey Botanical Club 112:187–193.

Nilsen E.T. 2002. Ecological relevance of allelopathy: Some considerations related to Mediterranean, subtropical, temperate, and boreal forest shrubs. Pp. 109–129, *In* Inderjit and A.U. Mallik. (Eds). Chemical Ecology of Plants: Allelopathy in Aquatic and Terrestrial Ecosystems. Birkhauser Verlag. Switzerland.

Nilsen, E.T., J.F. Walker, O.K. Miller, S.W. Semones, T.T. Lei, and B.D. Clinton. 1999. Inhibition of seedling survival under *Rhododendron maximum* (Ericaceae): Could allelopathy be a cause? American Journal of Botany 86:1597–1605.

Nilsen, E.T., B.D. Clinton, T.T. Lei, O.K. Miller, S.W. Semones, and J.F. Walker. 2001. Does *Rhododendron maximum* L. (Ericaceae) reduce the availability of resources above and belowground for canopy tree seedlings? American Midland Naturalist 145:325–343.

Peterson, E.B. 1965. Inhibition of black spruce primary roots by a water soluble substance in *Kalmia angustifolia*. Forest Science 11:473–479.

Rice, E.L. 1979. Allelopathy: An update. Botanical Review 45:15–109.

Rice, E.L. 1984. Allelopathy (2nd edition). Academic Press, New York, NY.

Swank, W.T., and D.A. Crossley. 1988. Introduction and site description. Pp. 3–17, *In* W.T. Swank and D.A. Crossley (Eds). Forest Hydrology and Ecology at Coweeta. Springer-Verlag, Berlin, Germany.

Walker J.F., O.K. Miller, T.T. Lei, S. Semones, E.T. Nilsen, and B.D. Clinton. 1999. Suppression of ectomycorrhizae on canopy tree seedlings in *Rhododendron maximum* L. (Ericaceae) thickets in the southern Appalachians. Mycorrhiza 9:49–56.

Waterman J.R., A.R. Gillespie, J.M. Vose, and W.T. Swank. 1995. The influence of mountain laurel on regeneration in pitch pine canopy gaps of the Coweeta Basin, North Carolina, USA. Canadian Journal of Forest Research 25:1756–1762.

Woods, F.W., and R.E. Shanks. 1959. Natural replacement of chestnut by other species In the Great Smoky Mountains National Park. Ecology 40:349–361.

Yamasaki S.H., J.W. Fyles, K.N. Eggers, and B.D. Titus. 1998. The effect of *Kalmia angustifolia* on the growth, nutrition, and ectomycorrhizal symbiont community of black spruce. Forest Ecology and Management 105:197–207.

Zhu, H., and A.U. Mallik. 1994. Interactions between Kalmia and Black spruce: Isolation and identification of allelopathic compounds. Journal of Chemical Ecology 20:407–421.