Rust-Enhanced Allelopathy of Perennial Ryegrass against White Clover

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ABSTRACT

Perennial ryegrass (Lolium perenne L.) and white clover (Trifolium repens L.) are important pasture components in the higher rainfall areas of southeastern Australia. Crown rust (Puccinia coronata Corda f.sp. lolii Brown) is the most serious ryegrass pathogen in these areas. In a preliminary investigation, rust reduced ryegrass biomass by 56%. Yet, interference from rusted ryegrass suppressed the yield of neighboring clover plants more than interference from healthy ryegrass. The role of allelopathy in this relationship was investigated in a greenhouse study using two bioassays. Soil previously growing rusted ryegrass suppressed clover biomass by 36% compared with soil previously growing healthy ryegrass. Similarly, leachate from soil surrounding rusted ryegrass suppressed clover biomass by 27% compared with that from healthy ryegrass. This is the first demonstration that a pathogen may influence allelopathy between plants and that rust may enhance ryegrass allelopathy against clover. Possible implications of this in pasture ecology and the evolution of mutualism are discussed.

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Connolly, 1990). Crown rust is the most serious ryegrass fungal pathogen in these areas (Eagling and Clark, 1993), with epidemics regularly occurring between spring and autumn. A preliminary study demonstrated that rust accelerates senescence and reduces ryegrass yield by 56% (Mattner, 1998). Despite this, in ryegrass and clover mixtures, interference from rusted ryegrass suppressed clover biomass by up to 47% compared with interference from healthy ryegrass. This suppression did not result from a direct effect of crown rust on clover, because rust-inoculated and non-inoculated clover monocultures yielded the same, which was expected since clover is a nonhost of crown rust. Similarly, clover suppression is not explained by rust increasing ryegrass competitiveness because, if this were so, the reduction in clover yield would be greatest at high densities where resources are most limited. Instead, clover suppression was greatest at low densities, where competition for resources was minimal. Ryegrass allelopathy is well documented, particularly against clovers and medic (Gussin and Lynch, 1981; Takahashi et al., 1988, 1991, 1993; Quigley et al., 1990; Chung and Miller, 1995). For these reasons, we investigated the hypothesis that rusting increases ryegrass allelopathic ability.

Both Rice (1984) and Einhellig (1995) hypothesized that pathogens enhance their host’s allelopathic ability. Evidence supporting this hypothesis occurs in at least two forms. Firstly, pathogens stimulate phytoalexin (antimicrobial compounds) production by their hosts (Smith, 1996), which can belong to similar chemical groups and are synthesized via the same biochemical...
pathways as allelochemicals. For example, isoflavonoids are important phytoalexins (Dakora and Phillips, 1996) and allelochemicals (Tamura et al., 1967, 1969) from the Leguminosae. Indeed, many phytoalexins act as allelochemicals against plants, inhibiting their germination (Chang et al., 1969), growth (Glazener and Van-En etten, 1978), and cellular metabolism and function (Gi-annini et al., 1990; Spessard et al., 1994). Secondly, under some conditions, the mutualistic fungus Neotyphodium lolii (Latch, Christensen and Samuels) Glenn, Bacon and Hanlin, increases the allelopathic ability of some ryegrass genotypes (Sutherland and Hoglund, 1990; Quigley et al., 1990). Despite this, no one has previously observed that diseased plants suppress the growth of neighboring species (Tang et al., 1995), even though the effect of pathogens on interference between host and nonhost plants has been extensively investigated (Bur- don, 1987; Ayres and Paul, 1990). Since competition is the dominant process of plant interference (Tilman, 1988), competition effects often obscure any allelopathic effects in such experiments (Trenbath, 1974). This is particularly so at high plant densities where competition is intense. While it is difficult to separate the processes of competition and allelopathy in the field, separation can be achieved using bioassays. To date, however, re-searchers have not used bioassays to investigate the effects of pathogens on allelopathy.

The present investigation examined the hypothesis that rust enhances perennial ryegrass allelopathy against white clover using two bioassay techniques.

**METHODS AND MATERIALS**

**Pathogen and Plant Material**

In the following bioassays, perennial ryegrass (cv. Victor- ian) was used as the donor species and white clover (cv. Tamar) was used as the receiver species. The perennial ryegrass cultivar Victorian is highly susceptible to crown rust (Critchett, 1991). Crown rust urediniospores were collected with a side-arm flask collector from naturally infected ryegrass at the Mt. Derrimut field station, 22 km west of Melbourne, Australia (37°47’ S, 144°47’ E) (Mattner, 1998).

**Growing Conditions and Soil Type**

Bioassays were conducted in temperature-controlled glass-houses (20–23°C during the day and 12–15°C at night) at the Mt. Derrimut field station. Pots were drip-irrigated at the rate of 150 mL of water twice daily, which maintained soil at near field capacity. The soil mixture was three parts red-brown earth topsoil to one part sand and two parts peat moss. The following nutrients (g/L) were added to the mixture: 0.200 N, 0.116 P, 0.140 K, 0.086 S, 0.652 Ca, 0.024 Cu, 0.012 Zn, 0.008 Mn, 0.028 Fe, 0.012 Mo, 0.360 Mg, and 0.001 B. The mixture was then pasteurized by steam prior to planting. Following pasteurization, soil used to grow white clover was inoculated with the appropriate strain of Rhizobium trifolii Dangeard. This was performed by watering soil contained in individual pots with 150 mL of an inoculum solution (10 g of commercial inoculum per 10 L of water).

**Soil Retrieval Bioassay**

Pots (15 cm diam.) containing the standard soil mix were sown to contain an average of 10 ryegrass donor plants. In total there were 108 pots, half with rusted ryegrass and half with nonrusted ryegrass. Plants in rusted treatments were spray inoculated (Villalta and Clarke, 1995) 52 d after sowing with an aqueous solution containing 1 × 10⁶ urediniospores/mL, 0.1% soft soap as a surfactant, and 0.5% gelatine. Plants in nonrusted treatments were sprayed with a similar solution containing no spores. Following inoculation all plants were placed in plastic tents for 1 wk, which maintained humidity between 92 and 98%. Donor plants were grown for a total of 100 d.

Soil for the bioassay was gathered directly from the pots used to grow rusted and nonrusted donor ryegrass, all of which was removed by hand and then passed through a 5-mm sieve. Soil from each source was bulked and thoroughly mixed prior to further treatment. A fresh preparation of soil was also included as a control. In addition, plus and minus nutrient and steam-sterilization treatments were incorporated into the design. This was done in an attempt to detect confounding effects caused by differences in soil microflora and nutrient content in the soils resulting from prior rusted and nonrusted ryegrass growth. Nutrient enrichment consisted of adding concentrations of the nutrients listed previously, to ensure they were slightly in excess of growth requirements. The soils were then placed into 12.5-cm-diam. pots and used to grow two clover receivers. At 70 d after sowing, receivers were washed free of soil and dried at 80°C for 4 d before total biomass was determined. Additionally, a nodulation index was determined by bunching individual plant roots; removing three 2-cm sections from the upper, central, and lower portion of the bunch; counting the nodules captured in each section; drying each sample; and recording the number of nodules per gram of root.

The bioassay was conducted as a randomized complete factorial design with three blocks. There were four pots per treatment in each block. Factors consisted of soil source (three levels: soil previously growing rusted ryegrass, soil previously growing nonrusted ryegrass, and freshly prepared soil as a control), nutrient application (two levels: plus and minus) and soil sterilization (two levels: plus and minus).

**Soil Leachate Bioassay**

Pots (15 cm in diam.) containing the standard soil mix were sown to contain eight ryegrass donor plants. Twenty days after sowing, irrigation lines were placed into individual pots and calibrated to deliver 250 mL of water per pot every day. Soil leachate was collected in plastic trays beneath the wire mesh benches, bulked for each treatment, and thoroughly mixed prior to application to receiver plants. Seventy days after sowing, plants in the rusted treatment were inoculated with rust as described previously. One-half of the 64 pots contained rusted ryegrass and the other half nonrusted plants.

The investigation was conducted in two parts. In the first, the pre-inoculation bioassay, the bioassay was made before donor plant inoculation to ensure that there were no intrinsic differences in the allelopathic potential of donor plants within the rust treatments prior to inoculation. The second, the postinoculation bioassay, was made after inoculation when rust symptoms had fully developed. The methodology of the bio-assays was the same. Pots (12.5 cm in diam.) filled with the standard soil mix were prepared containing two clover receivers. Each was hand watered daily with 150 mL of the appropriate leachate or with water in the case of the controls.

Receivers were harvested and compared 50 d after sowing by determining plant biomass, leaf area (with a planimeter, Paton Industries Pty. Ltd., South Australia), leaf number, stolon number, and nodulation index. Measurements were taken
on one randomly selected plant per treatment in each block, except for biomass where all plants were measured.

The bioassay was conducted as a randomized complete block design. The treatments were the daily application of 150 mL of leachate derived from either rusted ryegrass, nonrusted ryegrass, or irrigation water. There were eight blocks consisting of four pots per treatment.

### Statistical Analysis

Data was analyzed using analysis of variance (ANOVA) as performed on Minitab Version 12 (Minitab, 1998). Homogeneity of variance was determined by examining plots of fitted values versus residuals, while histograms of residuals assessed normality of distribution.

### RESULTS

#### Soil Retrieval Bioassay

Table 1 presents statistical significance levels of main treatments and their interactions in influencing clover biomass and nodulation. Sterilization of the soils used in this bioassay had no effect on the biomass of clover growing in them. As such, sterilization treatments have been grouped to provide a clearer demonstration of the effects of the different soil source and nutrient treatments (see Fig. 1). The biomass of clover grown in soil from rusted ryegrass was less than that in the control or in soil from nonrusted ryegrass, with this effect being particularly marked in nutrient treated soils. In contrast, clover grown in soil from nonrusted ryegrass produced less than the control only when no nutrients were added.

Although soil sterilization did not affect clover biomass, it reduced root nodulation by 26% (see Table 2).

### Soil Leachate Bioassay

The pre-inoculation bioassay detected no difference in the growth of clover watered with leachate from ryegrass in nonrusted or rusted treatments (see Table 3). This indicates that there was no intrinsic difference in the allelopathic ability of ryegrass plants assigned to the treatments prior to inoculation. Growth in the control, however, was greater than that of plants receiving soil leachate from ryegrass.

In the post-inoculation bioassay, the growth of clover receiving soil leachate from rusted ryegrass was less than that of plants watered with leachate from nonrusted ryegrass or the control, according to each parameter measured (see Table 3). Plants exposed to leachate from nonrusted ryegrass did not differ from the control in any way. Nodulation did not vary between treatments.

### DISCUSSION

Even though several authors have suggested that pathogens increase their host’s allelopathic ability (Rice, 1984; Einhellig, 1995), the present study is the first to evaluate the pathogen effect on allelopathy between plants. Furthermore, no one has previously observed that diseased plants suppress the growth of neighboring species (Tang et al., 1995). Despite this, a preliminary investigation found that rusted ryegrass suppressed the growth of neighboring clover plants by up to 47% compared with when grown with healthy ryegrass (Mattner, 1998).

In the present study, the soil retrieval bioassay demonstrated that, overall, soil previously growing ryegrass reduced the subsequent clover growth compared with...
a control of freshly prepared soil. The modification of soil microflora by ryegrass to contain species antagonistic to clover growth does not explain this result because soil sterilization had no effect on the relationship. In contrast, nutrient addition alleviated the suppressive effect that soil previously growing healthy ryegrass had on clover production. This suggests that the main effect of healthy ryegrass was to deplete soil nutrients and thereby diminish clover growth. This was not the case for soil previously growing rusted ryegrass, however, where nutrient application markedly increased its suppressive effect on clover growth. Under these conditions, soil previously growing rusted ryegrass suppressed clover biomass by 36% compared with soil previously growing healthy ryegrass. This was in similar proportions to that in the preliminary experiment where the yield of clover grown in mixtures with rusted ryegrass fell by an average of 37% (Mattner, 1998). Nutrient toxicity does not explain this result because twice the concentration of nutrients applied to soil in the rusted treatment had no effect on clover growth in the control. Instead, the result provides strong evidence that rust increases ryegrass allelopathic ability against clover. Furthermore, the above results demonstrate a potential for rusted ryegrass to release allelochemicals into soil at concentrations phytotoxic to clover.

Previous soil-based bioassays have not reported enhanced allelopathy following nutrient addition (Buchholtz, 1971). Despite this, there are at least three possible explanations of how nutrients might enhance clover suppression by soil previously growing rusted ryegrass:

(i) The presence of abundant nutrients in some way facilitates allelochemical uptake.

(ii) Nutrient addition may initiate cationic exchange. Here, nutrient addition in the form of cations releases allelochemicals bound to anionic colloidal or organic material into the soil solution, where they are absorbed by receiver plants.

(iii) A reaction of allelochemicals with the added nutrients may increase their toxicity and/or stability.

Takahashi et al. (1988, 1991) established that leachate from perennial ryegrass can be phytotoxic to white clover. In a similar manner, the present study showed that soil leachate from developing ryegrass (up to 70 d after sowing) suppressed clover biomass by 30% compared with the control (i.e., in the pre-inoculation bioassay). This highlights the potential for ryegrass to suppress clover through allelopathy. As healthy ryegrass developed, however, the phytotoxicity of its leachate diminished and was lost, suggesting that ryegrass allelopathic potential is greatest when it is young. Work on other plant species has also shown a similar decrease in allelopathy as plants age (Koeppe et al., 1970; Woodhead, 1981). The hypothesis that allelopathic potential decreases with plant age may also explain why Newman and Rovira (1975) and Newman and Miller (1977) observed no clover yield depression following exposure to leachate from ryegrass. This is because they used leachate only from mature ryegrass, starting at 60 d after sowing.

In contrast to leachate from healthy ryegrass, plant age did not affect the phytotoxicity of leachate from rusted ryegrass, where the leachate suppressed clover biomass by 20% compared with the control and by 27% compared with nonrusted ryegrass. This is again in similar proportions to the preliminary investigation (Mattner, 1998). Similar reductions occurred in all parameters measured in clover exposed to leachate from rusted ryegrass, strongly supporting the hypothesis that rust increases ryegrass allelopathic ability against clover. The results also suggest that in addition to enhancing ryegrass allelopathic potential, rust prolongs allelopathy well into maturity.

While results from each bioassay suggest that rust can increase ryegrass allelopathy against clover, there was no evidence of allelopathy acting against nodulation. Furthermore, the uniformity of size and pinkness of nodules from plants in all treatments suggested that allelopathy did not impair nodulation or nitrogen fixed capacity, although no measurement of this was made. The only treatment that affected nodulation was soil sterilization, which reduced it by 26%. This was probably due to sterilization reducing the resident rhizobial population in the soil prior to planting.

The ability of rust to increase ryegrass allelopathic potential is not surprising given that infection by pathogens can induce phytoalexin production by their hosts (Smith, 1996). Phytoalexins can belong to similar chemical groups as allelochemicals. For example, Mayama et al. (1981, 1982) discovered that rust resistant oat (Avena sativa L.) produced three phytoalexins belonging to the phenolic acid group when challenged by crown rust. These phenolic acids inhibited rust spore germination at concentrations as low as 200 mg/L. Although these chemicals were not shown to suppress plant growth, they have a great potential to do so given that many allelochemicals are phenolic acids.

Another potential allelochemical source from rusted ryegrass in the present experiment is from the rust itself. It is well established that pathogens can produce a range of phytoxins that are important in pathogenesis (Daly and Deverall, 1983). Despite this, a preliminary investigation demonstrated that clover monoculture inoculation with crown rust had no effect on its growth (Mattner, 1998). This suggests that the allelochemical source from rusted ryegrass in the present experiment is more likely to be from ryegrass than from rust. It is important to note, however, that irrespective of the phytoxin source from rusted ryegrass, the relationship between rusted ryegrass and clover remains allelopathic. This is because rust is a biotrophic parasite of ryegrass and allelopathy is defined as the beneficial and detrimental chemical interaction among plant organisms, including microorganisms (Rice, 1984).

An important next step in the confirmation of enhanced allelopathy by rusted ryegrass is to identify the allelochemicals involved. What is their source? Are these compounds in higher concentrations in rusted plants or do rusted plants produce entirely different
allellochemicals than healthy plants? In a related question, what is the effect of disease severity on ryegrass allelopathic ability? The average disease severity of rusted ryegrass at 42 d after inoculation in the soil retrieval bioassay of the present experiment was 6.6% (as determined by a calibration method [Mattner and Parbery, 1999]). If rust enhances allelopathy between ryegrass and clover, heavily rusted plants should produce more allelochemicals than lightly rusted plants. Moreover, the ryegrass cultivar used in the present experiment was highly susceptible to rust. If rust enhances ryegrass allelopathy through phytoalexin production, rust resistant cultivars might produce more phytoalexins, or allelochemicals, than susceptible cultivars.

The increased allelopathy of rusted ryegrass has important implications, both for pasture ecology and the evolution of mutualism. In Australia, rust epidemics occur in ryegrass between late spring and early autumn. During this period, relative white clover growth is greater than that of perennial ryegrass, due mainly to differences in temperature optima (18–21°C for ryegrass and 24°C for clover) for growth (Haynes, 1980). The combined effects of crown rust and increased competition from clover increase ryegrass mortality. For this reason, the increase in allelopathy by ryegrass following rust infection may contribute to preventing its eradication from pastures during the summer–autumn period. Although pathogens are largely detrimental to their hosts, they may also bestow some benefits (Parbery, 1996). By conferring some benefit on its host, the pathogen maintains a self-advantage through increasing the survival chances of its host and ultimately itself. Furthermore, Clay (1988) suggested that pathogens evolve toward a mutualistic relationship with their hosts through the acquisition of beneficial characteristics or “new functions.” Since biotrophic parasites such as the rusts are heavily dependent on the continuity of their host genotype into succeeding generations, the evolution of interactions that enhance the chances of host survival are important. It is well established that infections of fodder species by biotrophs can create conditions that either limit grazing of their hosts, limit the number of grazing animals, or both. Morgan and Parbery (1980) established that infection by *Pseudopeziza medicagnis* (Lib.) Sacc. lowered protein content, digestibility, and palatability of lucerne (*Medicago sativa* L.) as well as increasing its oestrogenic activity. Similarly, Critchett (1991) determined that crown rust reduces ryegrass digestibility and quality. For this reason, ruminants preferentially graze healthy ryegrass rather than rusted ryegrass (Cruickshank, 1957), indirectly benefiting rusted ryegrass. Evidence from the present study suggests that rust adds a further benefit to ryegrass, that of increased allelopathy with neighboring plants. In a similar manner to crown rust, amongst other benefits, the mutualistic endophyte *N. lolii* reduces ryegrass palatability to ruminants (Fletcher and Sutherland, 1993) and reportedly increases its allelopathic ability (Sutherland and Hoglund, 1990; Quigley et al., 1990). Is the pathogenic relationship between crown rust and ryegrass evolving toward mutualism?

### CONCLUSIONS

The bioassays of the present study showed that soil and leachate from rusted ryegrass suppressed clover growth compared with that from nonrusted ryegrass. Together, these bioassays provide strong evidence for the hypothesis that rust enhances perennial ryegrass allelopathic ability against white clover. This hypothesis would explain why rusted ryegrass suppressed the growth of neighboring clover plants in a preliminary experiment (Mattner, 1998).

### REFERENCES


Mattner, S.W., and P.G. Parbery. 1999. The interaction of endophyte...


