Resistance in Annual *Medicago* spp. to *Phoma medicaginis* and *Leptosphaerulina trifolii* and Its Relationship to Induced Production of a Phytoestrogen

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ABSTRACT

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Foliar and stem diseases of annual *Medicago* spp. caused by *Phoma medicaginis* and *Leptosphaerulina trifolii* can not only reduce yield, but also affect herbage quality by inducing the production of the phytoestrogen coumestrol. To determine differences in host reaction to these pathogens, 33 cultivars and lines in 1993 and 10 cultivars in 1995 were evaluated in inoculated field tests. In the 1993 test, a number of genotypes with high levels of resistance to leaf and stem disease caused by *P. medicaginis* and to leaf disease caused by *L. trifolii* were identified. Genotypes with very high levels of resistance to stem disease caused by *P. medicaginis* were *M. sphaerocarpos* GRC5659.4.1 and SAD10069, *M. murex* GRC87.1, GRC707, and GRC708, *M. truncatula* Z771, and *M. solerolii* DZA3180.1, all of which had stem disease scores of ≤ 1.0 (scale 0 to 10) by the end of the growing season. The levels of coumestrol produced ranged from 114 to 1,230 ppm dry weight in stems across the genotypes, and the score for stem disease caused by *P. medicaginis* in the corresponding cultivars ranged from ≤ 0.8 to 8.9, respectively. The 1995 test confirmed the relative responses of nine cultivars (Caliph, Circle Valley, Cyprus, Harbinger AR, Zodiac, Paraggio, Santiago, Serena, and Orion) of annual *Medicago* spp. to leaf and stem disease caused by *P. medicaginis* and to stem disease caused by *L. trifolii*. Those with the lowest levels of coumestrol in the stems were *M. solerolii* DZA3180.1, *M. truncatula* Paraggio, and *M. sphaerocarpos* SAD10069, all with levels ≤ 130 ppm. The highest level was found in *M. polymorpha* SA4178 (1,230 ppm). *M. littoralis* Harbinger AR, Z286, Z298, and Z912, *M. murex* 89F16.1.1, *M. orbicularis* SA8460, and *M. polymorpha* SA4188, all had coumestrol levels of >600 ppm. For stem disease caused by *P. medicaginis* in particular, there was significant correlation of the level of disease with the level of coumestrol in stems at the end of the growing season. In contrast, for *L. trifolii*, there was significant negative correlation (leaf disease) or only a weak positive correlation (stem disease) with coumestrol in stems at the end of the growing season. Incorporation of these identified disease resistances into commercial cultivars offers a promising avenue not only as a long-term strategy for management of foliar diseases in annual *Medicago* spp., but also as a means of reducing phytoestrogen levels in commercial annual *Medicago* spp. pastures in order to minimize the adverse effects of phytoestrogens on fertility levels in sheep.

Stem and leaf spots caused by one or more *Phoma* and *Leptosphaerulina* species can cause significant losses to forage production in subterranean clover (*Trifolium subterraneum*) in southern Australia (4). *Phoma* black stem disease, caused by *Phoma medicaginis* and also known as spring black stem and leaf spot on alfalfa (26), is also an important fungal disease of *Medicago* species in Europe, North America, South Africa, and Australia (1,8,9,22, 24,25,31,33,34). In Western Australia, it is a major problem in the second year after seeding in most annual *Medicago* spp.

stands, and poses a potential threat to susceptible cultivars (2,11,24), especially in rainfall areas of >350 mm per year with long pasture phases between cereal crops (10–12). Yield losses can be substantial. Reductions in herbage production of up to 16% and seed yield of up to 20% have been reported in grazed swards, while losses of herbage and seed of up to 32 and 53%, respectively, have been reported in ungrazed swards (6,7). This disease can cause complete defoliation and premature death of very susceptible annual *Medicago* spp., particularly in lush stands during wet weather (1).

Pepper spot disease, caused by *Leptosphaerulina trifolii*, commonly occurs wherever annual *Medicago* spp. are grown, including Europe, South Africa, North America, and Australia (4,8,9,22, 25,27,29,32). In Australia, it can cause substantial yield losses in susceptible cultivars (12).

Identification of host resistance to necrotrophic foliar pathogens has frequently been a key aim of annual *Medicago* spp. cultivar development programs, especially as there is large variability within annual *Medicago* spp. for resistance to diseases such as *Phoma* black stem disease (2,3,5,7,31,32,36). Some resistance to stem and leaf disease of annual *Medicago* spp. has previously been identified for pepper spot disease, both in Australia (12) and in North America (23,32).

Diseases such as *Phoma* black stem are also known to stimulate production of phytoestrogenic compounds such as coumestrol (10,12,15,21) on annual *Medicago* spp. to levels between 300 and 550 ppm in stems and/or burrs in ungrazed swards, and levels in excess of 1,000 ppm have been recorded for *M. tornata* cv. Swani (7,8,10,12). However, in all these studies, only the level of coumestrol was assessed, and other coumestans such as 4'-*O*-methyl coumestrol (14) were not included, as coumestrol is the most easily measured of these phytoestrogenic compounds. While phytoestrogens may provide some advantage to the host in terms of providing protection against attack by pests, significant amounts of phytoestrogenic compounds (34), perhaps even levels as low as 25 ppm dry weight in annual *Medicago* spp. pastures (16), can affect ovulation rates in sheep. A previous survey of annual *Medicago* spp. pastures in Western Australia indicated that very high levels of both foliar disease and coumestrol can sometimes occur together (16–19). Response to disease varies between genotypes for the quantity of phytoestrogens produced (10,12). The severe losses from *P. medicaginis* and *L. trifolii* in susceptible annual *Medicago* spp. and the coumestrol stimulation associated with this infection (12) emphasize the need for future cultivars to carry resistance to both the pathogens as well as a low predisposition to coumestrol production.

This paper reports field screening of advanced breeding lines and/or cultivars of eight different annual *Medicago* spp. to identify resistance to *P. medicaginis* and *L. trifolii* and also to evaluate the relationship of resistance to disease within these genotypes with stimulation of coumestrol production in host tissues in response to these two pathogens.

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MATERIALS AND METHODS

Field tests. Field tests were conducted in 1993 and 1995 to determine the reaction of genotypes and cultivars of annual *Medicago* spp. to *P. medicaginis* and *L. trifolii* and the accumulation of the phytoestrogen coumestrol. The test genotypes and cultivars were sown by hand at 40 kg/ha in 0.8 × 0.8 m plots at the Department of Agriculture and Food Western Australia's Medina Experimental Station (latitude 32° 13' S; longitude 115° 48' E), Medina, Western Australia, on 6 May 1993 or 9 May 1995 by spreading seed over the plot surface and lightly raking to cover the seed with soil. The soil type was a yellow sandy soil with a Northcote Convention code of Uc4.2 (30). Seed was inoculated with an appropriate rhizobial strain, and seed was lime pelleted prior to sowing in order to protect the rhizobia coated onto the seed. Approximately 200 kg/ha of metham sodium was applied to the soil using commercial soil-injection equipment 5 weeks prior to sowing, and the fumigated area was then watered by overhead sprinklers immediately following application of the metham sodium to minimize escape of the fumigant into the atmosphere. Fertilizer applications were according to standard practice and included superphosphate at 200 kg/ha and muriate of potash at 50 kg/ha topdressed prior to seeding. Subsequently, a 4:1 ratio mix of superphosphate and muriate of potash was applied at the end of June and again in late August. Dimethoate at 60 ml/ha was applied at the end of June and again at the end of August to control any redlegged earth mites (*Halotydeus destructor*) and/or aphid species present. All plots were watered as required using overhead sprinklers, and were surrounded by a 1-m bare ground buffer.

In 1993, 33 annual *Medicago* spp. genotypes and cultivars, *M. murex*, Zodiac, 89FO5.5.1, 89FB6.1.1.3, 89F16.1.1, 89F17.4.3, GRC707, GRC708, GRC87.1; *M. sphaerocarpos*, 89N19.5.1.1, GRC5659.4.1, SAD10069, Orion; *M. polymorpha*, Serena, Santiago, Circle Valley, SA4178, SA4188, SA5709, SA21255; *M. orbicularis*, SA8460; *M. tornata*, MCD39C; *M. littoralis*, Z905, Z245, Z286, Z298, Z912, Harbinger AR; *M. truncatula*, Paraggio, Mogul, Z771, Caliph, Cyprus; and *M. solerolii*, DZA3180.1, were examined. Seed of cultivars was obtained from commercial certified seed, and seed of advanced breeding lines was obtained from the Australian *Medicago* Genetic Resource Centre, South Australia. Genotypes were replicated four times in a randomized block design.

In 1995, 10 cultivars of annual *Medicago* spp., Caliph, Circle Valley, Cyprus, Harbinger AR, Herald, Zodiac, Paraggio, Santiago, Serena, and Orion, were examined to confirm their relative responses to *P. medicaginis* and to *L. trifolii*. Cultivars were replicated four times in a randomized block design.

Pathogen inoculation and disease assessment. Senesced annual *M. polymorpha* plants infected with both *P. medicaginis* and *L. trifolii*, collected at the end of the 1992 growing season from infested commercial stands of annual *Medicago* spp., was used in both the 1993 and 1995 tests. Following collection, inoculum was stored at room temperature until needed, and was distributed by hand at 830 kg/ha across each plot on 13 May 1993 (1993 test) or 15 May 1995 (1995 test).

Once disease development had become evident, plots were assessed at approximately 4 weekly intervals throughout the season for any fungal diseases present. In 1993, assessments for leaf disease caused by *P. medicaginis* and *L. trifolii* were made on 15 June, 12 July, 10 August, and 8 and 30 September. In 1995, assessments for leaf disease caused by *P. medicaginis* and *L. trifolii* were only made on 8 August. In both tests and for all disease assessments, independent visually assessed scores for each plot were made by two different assessors, and these two scores were averaged to present a single assessment per genotype.

Leaf disease was assessed by decile rankings of affected leaf number on a 0 to 10 scale, where 0 = no disease, 1 = >1 to 10, 2 = >10 to 20, 3 = >20 to 30, 4 = >30 to 40, 5 = >40 to 50, 6 = >50 to 60, 7 = >60 to 70, 8 = >70 to 80, 9 = >80 to 90, 10 = >90% of leaves affected. In 1993, only data for the 10 August assessment are presented, as this is the date when the disease scores for leaf disease caused by either *P. medicaginis* or *L. trifolii* peaked during the assessment period. The leaf symptoms of the diseases were clearly visually distinguishable and confirmed by occasional isolation onto moist blotter and/or nutrient agar plates.

For stem disease caused by *P. medicaginis* and *L. trifolii* in 1993, plots were assessed on 10 August, 8 and 30 September, and 27 October. Only data for the 27 October assessment are presented, as this is the date when the stem disease scores peaked for both diseases. The reason for assessments for stem disease caused by *P. medicaginis* commencing on 10 August while those for stem disease caused by *L. trifolii* commenced on 8 September was due to the latter disease being slower to become evident in plots. For the 1995 test for stem disease caused by *P. medicaginis*, plots were assessed on 7 and 27 September (only data for 27 September is presented), while for stem disease caused by *L. trifolii*, a single assessment was made on 27 October.

Stem disease was assessed by decile rankings of affected stems on a 0 to 10 scale where 0 = no disease, 1 = >0 to 10% of stems with small lesions; 2 = >10 to 20% of stems with small lesions; 3 = >20 to 30% of stems with lesions; 4 = >30 to 40% of stems with lesions; 5 = >40 to 50% of stems with lesions; 6 = >50 to 60% of

stems with mostly severe lesions; 7 = >60 to 70% of stems with severe lesions; 8 = >70 to 80% of stems with severe lesions and occasional stems collapsing from disease; 9 = >80 to 90% of stems with severe lesions and some stems collapsing; and 10 = >90 to 100% of stems collapsed from disease. The stem symptoms of the diseases were easily distinguished visually (dark brown to black lesions for *P. medicaginis* and small black "pepper spot" spots and/or lesions with light brown to tan centers for *L. trifolii*) and were also confirmed by occasional isolation onto moist blotter and/or nutrient agar plates. Each plot was scored by two assessors and the mean score used for analysis.

In both 1993 and 1995, stem samples were taken in early November from all plots for analysis of the content of the phytoestrogen coumestrol. Samples were oven dried at 70°C, finely ground using a coffee grinder, and assayed by thin-layer chromatography using a method based on Beck (13) and Francis and Millington (20). The method is semi-quantitative, and the values presented were calculated in comparison to standards of known coumestrol concentration.

Data analysis. Data were analyzed using Genstat (7th ed., Lawes Agricultural Trust, Rothamsted Experimental Station). Significant differences among means were assessed by least significant difference (LSD). Regression analysis was used to determine the relationship of *P. medicaginis* and *L. trifolii* disease scores to the level of coumestrol in stems.

RESULTS

1993 Field test. A number of genotypes with high levels of resistance to leaf disease caused by *P. medicaginis* were identified, including *M. littoralis* Z905 and *M. truncatula* Paraggio and Z771, both with scores of <1.0 by end of season (Table 1). This is in contrast to *M. polymorpha* Serena, Santiago, and Circle Valley, *M. murex* 89FB6.1.1.3, and *M. sphaerocarpos* Orion, all with leaf disease scores ≥2.9 by end of season.

A number of genotypes with very high levels of resistance to stem disease caused by *P. medicaginis* were identified, including *M. sphaerocarpos* GRC5659.4.1 and SAD10069, *M. murex* GRC87.1, GRC707, and GRC708, *M. truncatula* Z771, and *M. solerolii* DZA3180.1, which all had stem disease scores of ≤1.0 by the end of the growing season (Table 1). This is in comparison with other genotypes that showed high stem disease scores, such as cultivars *M. polymorpha* Serena (score 4.9) and Santiago (score 4.3), and some highly susceptible breeding lines such as *M. polymorpha* SA4178, SA4188, and SA21255 (scores ≥8.9) and *M. orbicularis* SA8460 (score 7.0).

The majority of genotype lines tested were highly susceptible to leaf disease

caused by *L. trifolii* (scores 3.2 to 5.5) (Table 1). The lines with the least disease were *M. littoralis* Z245, Z286, Z298, and Z905, and *M. polymorpha* SA4178, SA4188, and SA5709, all with leaf disease scores of ≤ 3.5 .

A number of genotypes showed no stem disease whatsoever caused by *L. trifolii*, including *M. murex* Zodiac and GRC708, *M. orbicularis* SA8460, *M. polymorpha* Circle Valley, Serena, Santiago, SA4178, and SA5709, *M. solerolii* DZA3180.1, *M. sphaerocarpos* Orion, GRC5659.4.1, SAD10069, and 89N19.5.1.1 (Table 1). The most susceptible genotypes to stem disease caused by *L. trifolii* were *M. truncatula* Caliph and Cyprus and *M. littoralis* Z298 and Z905, all with stem disease scores of ≥ 1.8 by the end of the growing season.

While there was no overall correlation between the leaf disease scores for *P. medicaginis* at 10 August with the stem disease scores for *P. medicaginis* at 27 October ($r = 0.12$, $n = 32$, ns), there was significant negative correlation between the leaf disease scores for *L. trifolii* at 10 August and stem disease scores for *L. trifolii* at 27 October ($r = -0.42$, $n = 32$, $P < 0.05$).

Levels of coumestrol produced in inoculated field swards ranged from 114 to 1,230 ppm across the genotypes (Table 1). Those with the lowest levels of coumestrol were *M. solerolii* DZA3180.1, *M. truncatula* Paraggio, and *M. sphaerocarpos* SAD10069, all with coumestrol levels ≤ 130 ppm. An extremely high level was found in *M. polymorpha* SA4178 (1,230 ppm). *M. littoralis* Harbinger AR, Z286, Z298, and Z912, *M. murex* 89F16.1.1, *M. orbicularis* SA8460, and *M. polymorpha* SA4188 all showed high levels of coumestrol at >600 ppm.

For leaf disease caused by *P. medicaginis*, there was significant positive correlation of the incidence of leaf disease at 12 July and at 30 September, but not at 15 June, 10 August, or at 8 September, with the level of coumestrol in stems at the end of the growing season (Table 2).

For leaf disease caused by *L. trifolii*, there was significant negative correlation of leaf disease score at 15 June, at 12 July, at 10 August, and at 8 September, but not at 30 September, with the level of coumestrol in stems at the end of the growing season (Table 2).

For stem disease caused by *P. medicaginis*, there was significant correlation of the score for stem disease at 10 August, at 8 September, at 30 September, and at 27 October with the level of coumestrol in stems at the end of the growing season (Table 2).

For stem disease caused by *L. trifolii*, there was a weak positive correlation of stem disease score at 8 September but no significant correlation of stem disease at 30 September, nor at 27 October, with the

level of coumestrol in the stems at the end of the growing season (Table 2).

1995 Field test. For leaf disease caused by *P. medicaginis*, *M. murex* Zodiac had the least amount of disease, with a disease score of 0.8. *M. truncatula* Cyprus and *M. littoralis* Harbinger appeared to be the next most resistant with disease scores ≤ 1.8 (Table 3). The most susceptible cultivars were *M. polymorpha* Santiago, Serena, and Circle Valley, all with disease scores of ≥ 5.0 .

For leaf disease caused by *L. trifolii*, *M. polymorpha* Circle Valley had the lowest disease score of 0.9 followed by *M. polymorpha* Serena and *M. littoralis* Herald

with scores ≤ 2.0 (Table 3). The most susceptible cultivars were *M. truncatula* Caliph, *M. murex* Zodiac, and *M. littoralis* Harbinger AR, all with scores ≥ 5.0 .

For stem disease caused by *P. medicaginis*, *M. truncatula* Paraggio had the lowest disease score of 0.7, followed by *M. littoralis* Harbinger, *M. sphaerocarpos* Orion, and *M. murex* Zodiac, all with disease scores ≤ 2.0 (Table 3). The most susceptible cultivars were *M. polymorpha* Serena and *M. truncatula* Cyprus, with disease scores ≥ 5.9 .

For stem disease caused by *L. trifolii*, overall disease scores were very low and there was no disease at all on *M. polymor-*

Table 1. 1993 Field test: Foliar disease scores for *Phoma medicaginis* or *Leptosphaerulina trifolii* on 33 annual *Medicago* genotypes grown in artificially infested and ungrazed plots at Medina, Australia, 1993^a

Medicago species and genotype	Leaf disease score (0-10 scale) ^b		Stem disease score (0-10 scale) ^b		Coumestrol (ppm dry wt)
	<i>P. medicaginis</i>	<i>L. trifolii</i>	<i>P. medicaginis</i>	<i>L. trifolii</i>	
<i>M. littoralis</i>					
Harbinger AR	1.1	3.6	3.0	1.4	605
Z245	1.3	3.4	3.4	1.3	490
Z286	1.0	3.4	4.3	1.5	678
Z298	1.6	3.0	4.3	1.8	695
Z905	0.8	3.4	3.4	1.9	583
Z912	1.0	3.9	4.5	1.6	760
<i>M. murex</i>					
Zodiac	2.1	3.8	0.3	0	329
89FB6.1.13	3.0	3.6	4.6	1.3	513
89FO5.5.1	2.7	4.7	2.9	0.6	518
89F16.1.1	2.2	5.0	1.4	0.1	391
89F17.4.3	1.8	4.0	5.6	0	603
GRC87.1	2.1	4.9	0.9	0.2	279
GRC707	1.5	4.9	0.5	0.1	293
GRC708	1.5	5.3	0.2	0	204
<i>M. orbicularis</i>					
SA8460	1.7	4.4	7.0	0	718
<i>M. polymorpha</i>					
Circle Valley	2.9	3.6	3.0	0	490
Serena	3.0	3.6	4.9	0	476
Santiago	3.1	4.2	4.3	0	540
SA4178	1.6	3.2	8.9	0	1,230
SA4188	1.7	3.5	9.3	0.3	606
SA5709	2.4	3.2	2.1	0	308
SA21255	3.5	4.2	8.9	0.01	748
<i>M. solerolii</i>					
DZA3180.1	1.4	5.4	0.3	0	114
<i>M. sphaerocarpos</i>					
Orion	3.5	4.1	1.4	0	315
GRC5659.4.1	2.0	5.0	0.2	0	190
SAD10069	1.7	4.0	0.3	0	130
89N19.5.1.1	2.6	3.9	2.4	0	590
<i>M. tornata</i>					
MCD39C	1.6	3.8	1.9	0.8	414
<i>M. truncatula</i>					
Caliph	1.2	3.9	5.9	2.3	416
Cyprus	1.0	4.1	5.1	1.8	425
Mogul	1.2	3.9	1.6	1.1	158
Paraggio	0.9	3.6	0.8	1.3	114
Z771	0.9	4.0	0.5	0.5	164
Mean	1.9	4.0	2.0	0.6	457
F prob. $P <$	0.001	0.001	0.001	0.001	0.001
LSD $P < 0.05$	0.98	0.89	1.37	0.45	217

^a Number of replications = 4, arranged in a randomized block design. Plots were artificially infected by adding *M. polymorpha* trash infested with both *Phoma medicaginis* and *Leptosphaerulina trifolii*.

^b Disease assessment is on a 0 to 10 decile scale where 0 = no disease and 10 = $>90\%$ of leaves affected by leaf disease or complete collapse of the sward for stem disease. Disease assessments were made for leaf disease on 10 August and stem disease on 27 October.

pha Circle Valley, Santiago, and Serena, *M. sphaerocarpos* Orion, or *M. truncatula* Paraggio.

Lowest levels of coumestrol were found in *M. truncatula* Paraggio (77 ppm) and in *M. truncatula* Caliph and Cyprus, *M. sphaerocarpos* Orion, and *M. murex* Zodiac, which all had coumestrol levels ≤ 272 ppm.

There was no significant correlation between disease and coumestrol level for the 1995 test.

Comparison of 1993 and 1995 tests. A comparison made between foliar disease

scores in the 1993 test and those in the 1995 test for the 10 cultivars in common showed that there was a significant degree of correlation between the scores of leaf disease caused by *P. medicaginis* ($r = 0.71$, $n = 9$, $P < 0.05$), the scores for stem disease caused by *P. medicaginis* ($r = 0.80$, $n = 9$, $P < 0.01$), and the scores for stem disease caused by *L. trifolii* ($r = 0.81$, $n = 9$, $P < 0.01$), but not for the scores for leaf disease caused by *L. trifolii* ($r = 0.06$, $n = 9$, ns). A comparison made between coumestrol levels in 1993 and those in 1995 for the same 10 cultivars showed that

there was no significant correlation ($r = 0.01$, $n = 9$, ns).

DISCUSSION

This study highlights the availability of resistance to *P. medicaginis* across one or more genotypes of *M. littoralis*, *M. murex*, *M. orbicularis*, *M. polymorpha*, *M. solerolii*, *M. sphaerocarpos*, *M. tornata*, and *M. truncatula*. In particular, a number of genotypes with very high levels of resistance to stem disease caused by *P. medicaginis* were identified, including *M. sphaerocarpos* GRC5659.4.1 and SAD10069, *M. murex* GRC87.1, GRC707, and GRC708, *M. truncatula* Z771, and *M. solerolii* DZA3180.1, which all had stem disease scores of ≤ 1.0 by the end of the growing season. In addition, a number of genotypes with high levels of resistance to leaf disease caused by *P. medicaginis* were identified, including *M. littoralis* Z905 and *M. truncatula* Paraggio and Z771. Importantly, a number of genotypes were identified that had no stem disease caused by *L. trifolii*, including *M. murex* Zodiac and GRC708, *M. orbicularis* SA8460, *M. polymorpha* Circle Valley, Serena, Santiago, SA4178, and SA5709, *M. solerolii* DZA3180.1, *M. sphaerocarpos* Orion, GRC5659.4.1, SAD10069, and 89N19.5.1.1. These new sources of resistance are especially suited to Mediterranean environments, where annual *Medicago* spp. are reliant upon having effective resistance against serious necrotrophic pathogens such as *P. medicaginis* and *L. trifolii* if they are to be productive (36). These sources of resistance could be useful both as parental materials for breeding better cultivars and, in some instances, perhaps directly released as new resistant cultivars (9). For providing practical benefits to farmers, the most important of these resistances is that expressed on stems against *P. medicaginis*, which is both the most damaging of the two diseases examined in the current study and also more damaging on stems rather than on leaves in terms of adverse impact upon herbage and seed yields (8,10). Annual *Medicago* spp. cultivar development programs targeting disease resistance, such as those developed for Phoma black stem disease in Australia (3,5,7), could be expanded. Incorporation of resistance into commercial cultivars offers the most promising long-term strategy for management of foliar diseases in annual medics (11,36).

Levels of coumestrol produced in the artificially inoculated field swards varied widely, ranging from 114 to 1,230 ppm across the genotypes tested. Some of the genotypes tested in the current study, such as *M. solerolii* DZA3180.1, *M. truncatula* Paraggio, and *M. sphaerocarpos* SAD10069, all with coumestrol levels ≤ 130 ppm and stem disease resulting from *P. medicaginis* of ≤ 0.8 , offer significant potential for reducing the levels of phy-

Table 2. Level of correlation between foliar disease from *Phoma medicaginis* or *Leptosphaerulina trifolii* with the level of coumestrol (ppm dry wt) in the stems of 33 annual *Medicago* genotypes grown in artificially infested and ungrazed plots at Medina, Australia, 1993^a

Foliar disease	Correlation (r=) with stem		
	Assessment date	Coumestrol level (ppm)	Significance (P<)
<i>Phoma</i> leaf disease	15 June	0.11	ns
	12 July	0.21	0.05
	10 August	0.16	ns
	8 September	0.08	ns
	30 September	0.31	0.001
<i>Leptosphaerulina</i> leaf disease	15 June	-0.20	0.05
	12 July	-0.26	0.01
	10 August	-0.31	0.001
	8 September	-0.27	0.001
	30 September	-0.15	ns
<i>Phoma</i> stem disease	10 August	0.42	0.001
	8 September	0.57	0.001
	30 September	0.70	0.001
	27 October	0.74	0.001
	8 September	0.18	0.5
<i>Leptosphaerulina</i> stem disease	30 September	0.15	ns
	27 October	0.13	ns

^a For all comparisons, $n = 131$.

Table 3. 1995 Field test: Foliar disease scores for *Phoma medicaginis* or *Leptosphaerulina trifolii* and coumestrol levels in stems (ppm dry wt) for 10 annual *Medicago* genotypes grown in artificially infested and ungrazed plots at Medina, Australia, 1995^a

<i>Medicago</i> species and cultivar	Leaf disease score (0-10 scale) ^b		Stem disease score (0-10 scale) ^b		Coumestrol (ppm dry wt)
	<i>P. medicaginis</i>	<i>L. trifolii</i>	<i>P. medicaginis</i>	<i>L. trifolii</i>	
<i>M. littoralis</i>					
Harbinger AR	1.8	5.4	1.6	0.4	838
Herald	2.5	2.0	3.9	0.8	1,209
<i>M. murex</i>					
Zodiac	0.8	5.5	2.0	0	272
<i>M. polymorpha</i>					
Circle Valley	5.0	0.9	4.3	0	464
Santiago	5.5	3.4	4.0	0	362
Serena	5.0	1.5	6.3	0	392
<i>M. sphaerocarpos</i>					
Orion	4.0	5.5	1.9	0	236
<i>M. truncatula</i>					
Caliph	2.8	5.0	4.8	0.6	242
Cyprus	1.5	4.9	5.9	0.6	235
Paraggio	3.2	3.8	0.7	0.1	77
Mean	3.2	3.8	3.5	0.3	433
F prob. P <	0.001	0.001	0.001	0.001	0.001
LSD P<0.05	1.52	1.40	1.50	0.26	250

^a Number of replications = 4, arranged in a randomized block design. Plots were artificially infected by adding *M. polymorpha* trash infested with both *Phoma medicaginis* and *Leptosphaerulina trifolii*.

^b Disease assessment is on a 0-10 decile scale where 0 = no disease and 10 = >90% of leaves affected by leaf disease or complete collapse of the sward for stem disease. Disease assessments were made for leaf disease on 8 August for both pathogens and on 1 October and 27 September for *Phoma* and *Leptosphaerulina* stem disease, respectively.

toestrogens in annual medic pastures, either by their direct deployment as cultivars or as parental material in annual *Medicago* spp. breeding programs. There were a number of genotypes that had coumestrol levels above 600 ppm and one with a level of 1,230 ppm. Actual levels of total coumestrol could be higher since not all types of coumestrol are measured with the assay method used in this and other studies (7,8,10,12,15,21). Phytoestrogenic compounds adversely affect ovulation rates in sheep (16,35), even coumestrol levels as low as 25 ppm, and the rates reported in our current and the earlier studies often substantially exceeded 25 ppm. Hence, while it is probable that the production of phytoestrogens may provide some advantage to the host by providing protection against attack by pests, the adverse effects of phytoestrogen on animal productivity more than offset any potential benefits from their presence.

For stem disease caused by *P. medicaginis*, there was significant correlation of the level of disease with the level of coumestrol in stems at the end of the growing season. Production of phytoestrogenic compounds in response to disease is known to vary between accessions of annual *Medicago* spp. (10,12), and the current study confirms this finding. Although a number of different types of stresses such as soil type (21) and nutrition (28) can also cause increases in coumestrol levels in annual *Medicago* spp., it is clear from the current study that there is a close relationship between the level of damage caused by *P. medicaginis* on stems and the phytoestrogen levels in stems. This suggests that annual *Medicago* breeders should focus in particular on selecting for high resistance to stem disease caused by *P. medicaginis*. Despite there being no significant overall correlation between coumestrol levels in the 1993 test and those in the 1995, some cultivars showed similar reactions for at least some of the disease components assessed (e.g., *M. truncatula* Cyprus and Paraggio) and/or also for coumestrol production (e.g., *M. polymorpha* Circle Valley and *M. truncatula* Paraggio) across both years. However, for some of the other cultivars at least, it appears that site and/or seasonal differences or other factors can also influence the relationship between the level of coumestrol with foliar disease caused by *P. medicaginis* and/or *L. trifolii*. The lack of overall relationship between the 1993 and 1995 coumestrol levels may also have been at least partially a consequence of the lack of relationship between disease and coumestrol level in the 1995 test. The current study, along with the earlier studies, suggests that sowing of more disease-resistant cultivars could reduce current levels of coumestrol in annual *Medicago* spp. swards in response to the presence of pathogens (9) and may be a useful way of

dealing with sheep fertility issues associated with high levels of phytoestrogens.

It is clear, for reasons of both reducing yield losses and phytoestrogen levels in annual *Medicago* spp. pastures, that all new annual medics released for regions where Phoma black stem disease in particular is common should possess adequate host resistance. In Australia, despite the identification of a significant number of sources of host resistance, breeders have not delivered this benefit to farmers by way of more disease resistant cultivars. Even where host resistance has been identified, it is clear that the mechanisms and/or genetic basis of such resistances have yet to be defined.

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