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(54) **Method for protecting grass using an endophyte**

Verfahren zum Schutz von Gräsern mittels eines Endophyten

Methode de protection des herbages utilisant un endophyte

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Description

TECHNICAL FIELD

[0001] This invention relates to fungal endophytes and combinations of endophytes with grass plants. More particularly the invention relates to endophytes which form combinations with perennial, annual and hybrid ryegrasses and some other related grasses. Even more particularly the invention relates to combinations having reduced toxicity to grazing livestock as compared to cultivars of endophyte/ryegrass combinations in common use whilst still retaining resistance against pests and/or abiotic stresses.

BACKGROUND ART

[0002] Fungal endophytes of the genus *Neotyphodium* (formerly *Acremonium*) infect a number of temperate climate Pooideae grasses. The *Neotyphodium* endophytes can produce alkaloids which are considered to confer degrees of pest and possibly disease protection upon the plants in which they naturally occur (Rowan and Latch, 1994; Blank and Gwinn, 1992). Resistance to drought conditions has also been claimed (Elberson and West, 1996). The *Neotyphodium* endophytes are vertically transmitted through the seed of the grasses and no natural horizontal transmission has been established (Leuchtmann, 1997).

[0003] Many of the predominating natural endophyte infections of improved grass cultivars used for pastoral agriculture production also cause significant animal disorders, for example fescue toxicoses (Stuedemann and Hoveland, 1988) and ryegrass-endophyte toxicosis (Fletcher et al., 1999). These may be complex toxic reactions by animals to alkaloids produced under a range of plant growth conditions. Significant economic loss within pastoral agriculture systems can occur due to such animal toxicoses. On the other hand presence of at least some endophytes may be essential for the competitive persistence of the chosen grass in a pasture (Elberson and West, 1996, Fletcher and Easton, 2000).

[0004] It has also been found that grass lines can be artificially infected with selected endophytes. Axenic cultures of endophytes can be used to infect grass seedlings, grown initially under sterile conditions (Latch and Christensen, 1985), which can then be selected for desirable qualities, and multiplied for commercial use. Three significant examples of this technology have been developed by AgResearch Ltd: GREENSTONE™ tetraploid hybrid ryegrass with ENDOSAFE™ endophyte (Tapper and Latch, 1999, NZ Patent 233083); various perennial and hybrid ryegrasses with AR1 endophyte (Fletcher, 1999); and tall fescue cultivars with MaxQ™ (Bouton et al., 2002, US Patent 6,111,170).

Ryegrass-endophyte toxicosis

[0005] Perennial ryegrass infected with its common wild-type endophyte, grown for both forage and turf, frequently produces compounds of the lolitrem sub-group of indole diterpenes in concentrations in herbage sufficient to cause the serious animal disorder known as ryegrass staggers. Lolitrem B is considered the most abundant active substance and concentrations in excess of about 2 ppm of herbage dry matter may result in clinical symptoms of ryegrass staggers in grazing sheep, cattle, deer and horses.

[0006] The same ryegrass-endophyte associations also produce ergovaline and perhaps other ergot alkaloids which are believed to cause other symptoms in grazing sheep, cattle, deer and horses commonly associated with the ryegrass-endophyte toxicosis syndrome. These symptoms may include hyperthermia in warm humid conditions as evidenced by increased rectal temperatures and respiration rates and depressed basal prolactin levels.

[0007] These responses are likely to be elicited at ergovaline concentrations in ryegrass pastures above 0.5 ppm. Ergovaline is also believed to be responsible for the depressed growth rates associated with the toxicosis syndrome. Increased faecal moisture and faecal soiling in sheep is also associated with ryegrass-endophyte toxicosis but causes have not been ascribed to any particular toxins.

[0008] The ryegrass staggers symptoms and overall effect of lolitrems may be enhanced by the presence in herbage of other toxins such as ergovaline.

[0009] Both lolitrem B and ergovaline concentrations tend to be higher in leaf sheath and seed heads of perennial ryegrass than in the roots or leaf blade. They also undergo seasonal variation with peaks in summer to autumn.

Enhanced plant protection with reduced toxicosis

[0010] Endophytes confer degrees of protection to host plants against biotic and abiotic stress. Some endophyte-derived alkaloids are known to be toxic or deterrent to insect pests. Peramine is a feeding deterrent for and lolitrem is toxic to Argentine stem weevil, (*Listronotus bonariensis*) (Rowan et al., 1990; Prestidge and Gallagher 1985). Ergovaline is deterrent to black beetle (*Heteronychus arator*) (Ball et al., 1997). Where these alkaloids are absent or in very low concentration in plants, infestation by such pests become a problem. Hence it can be seen from the above discussion

that it is desirable to have a ryegrass that has low mammalian toxicity but which also contains deterrent and/or insecticidal compounds to help avoid insect or other pest problems.

[0011] It is desirable to provide an endophyte which produces alkaloid compounds in herbage of a host plant in a manner such that the usual combinations and concentrations of alkaloids in herbage as generally consumed by grazing animals in common farming practice does not cause practical toxicosis symptoms. It is further desired to provide an endophyte which produces alkaloid compounds in herbage of a host plant that protects the grass from pasture and/or turf pests relative to equivalent endophyte-free grass.

[0012] It is desirable to provide an endophyte which does not produce detectable levels of toxins from the lolitrem group or ergovaline group.

[0013] It is a desirable to provide an endophyte from the genus *Neotyphodium* that, in combination with a host grass, gives superior pest protection for forage and/or turf uses compared to either equivalent endophyte-free grass or grass infected with common wild-type *Neotyphodium lolii.*

[0014] It is further desired to provide an endophyte which produces compounds from the class of janthitrem epoxides.

[0015] It is still further desired to address the foregoing problems or at least to provide the public with a useful choice.

[0016] AU 73853 91 (UNITED KINGDOM GOVERNMENT) 3 OCT 1991 discloses a method of inoculating herbage plants with an endophyte of the species *Neothyphodium lolii* to produce relatively high levels of peramine and relatively low levels of or no lolitrem.

[0017] Popay A J et al: Resistance to Argentine Stem Weevil in Perennial Ryegrass Infected with Endophytes Producing Different Alkaloids", PROCEEDINGS OF THE NEW ZEALAND PLANT PROTECTION CONFERENCE, 8 AUG 1995 discloses the resistance to Argentine stem weevil in perennial ryegrass infected by *Neophydium lolii* endophytes.

[0018] For the purpose of this specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

[0019] Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

DISCLOSURE OF INVENTION

[0020] The present disclosure provides an isolated endophyte of *N. lolii* species, selected from the group consisting of: AR37; AR40; variations in *N. lolii* species as exemplified by AR37; variations in *N. lolii* species as exemplified by AR40; and combinations thereof; AR37 and AR40 cultures deposited by AgResearch Limited (of Grasslands Research Centre, Tennent Drive, Private bag 11008, Palmerston North, New Zealand) on 23 May 2003 at the Australian Government Analytical Laboratories (AGAL) accession number NM03/35819 (AR37) and NM03/35820 (AR40).

[0021] The invention provides a method of protecting a host grass from biotic and abiotic stresses by artificially inoculating the host grass with a strain of endophyte of *Neotyphodium lolli* species, characterised in that the strain of endophyte of N lolli produces 11,12-epoxy-janthitrem G.

[0022] When the *N. lolii* species is in combination with a host grass, said endophyte will not produce sufficient levels of a compound or compounds to adversely affect the health and performance in grazing animals.

[0023] In the present invention, the endophytes described above preferably do not produce the hitherto known toxic alkaloids lolitrem B and ergovaline at levels of less than 2 ppm lolitrem B and less than 0.5 ppm ergovaline. Preferably the lolitrem B and ergovaline levels are at detection levels of less than 0.1 ppm of dry matter.

[0024] It is the understanding of the applicant that the 11,12-epoxy-janthitrem G compound confers protection from pest predation upon the host grass plants and the grass-dominant pasture or turf as a whole without causing toxicosis of practical significance.

[0025] Preferably, the host grass is a perennial, annual or hybrid ryegrass. Most preferably, these are selected from the species: *Lolium perenne; Lolium multlflorum; Lolium x hybridum.*

[0026] Preferably, the toxicosis which is avoided is ryegrass-endophyte toxicosis. Most preferably the toxicosis is caused by an ergovaline toxin or a lolitrem toxin or a combination of ergovaline and lolitrem toxins.

[0027] Preferably, the abiotic stress is a water deficit.

[0028] Preferably the endophyte culture, if used, is an axenic culture.

[0029] Preferably, the endophyte culture, if used, has the same characteristics with respect to taxonomic classification, plant infectivity, alkaloid production, animal performance, and plant protection properties as the endophyte itself.

[0030] The infection may be achieved by modifying the host grass by the breeding, crossing, hybridisation, selection, or genetic modification of grass.

[0031] Combinations of the endophyte and host grass produced by methods of the present invention may result in the grass having enhanced root growth and more tillers in comparison to a host grass without endophyte infection, including for example wherein the host grass is a Pooideae grass.

[0032] There is provided a combination of the endophyte or endophyte culture as described above, and a Pooldeae grass wherein the combination produces 11,12-epoxy-janthitrem G in the grass and not more than 0.1 ppm of ergovaline in the dry matter of whole herbage.

[0033] There is provided a combination of an endophyte as described above and a Pooideae grass wherein the combination has features selected from the group consisting of: enhancement of pest protection; resistance to insects; pasture persistence; and combinations thereof.

[0034] There is provided a combination of an endophyte as described above and a Pooideae grass wherein the combination has the features of enhancement of grazing animal growth, and increased animal productivity in comparison with grass infected with known endophytes capable of inducing the disorder known as ryegrass-endophyte toxicosis.

[0035] There is provided a combination of an endophyte or endophyte culture as described above and a host grass wherein the pest to which increased resistance is conferred on the host grass is selected from the group consisting of: root aphid (*Aploneura lentisci*); mealy bug (*Balanococcus poae*); Argentine stem weevil (*Listronotus bonariensis*); black beetle (*Heteronychus arator*); porina (*Wiseana cervinata*); and combinations thereof.

[0036] This disclosure provides seeds of a host grass infected with the endophyte as described above.

[0037] This disclosure provides the specific indole compound from the class of janthitrem epoxides produced from a host grass infected with the endophyte culture as described above.

[0038] The disclosure provides the use of a compound from the class of janthitrem epoxides as described above as a pesticide or as an insecticide.

[0039] Endophytes of the group of AR37 and AR40 may be identified by a method which includes

(a) growing seed, preferably from collections of grass seed;

(b) harvesting and drying samples of herbage;

(c) obtaining a solvent extract from the dried herbage;

(d) examining such solvent extracts for the purposes of determining the presence of compounds of the janthitrem class of indole diterpenes (as described below) and the absence of compounds of the lolitrem class of indole diterpenes and the absence of ergovaline at detection levels of 0.1 ppm of dry matter by procedures selected from the techniques of high pressure liquid chromatography; reverse-phase chromatography; flash chromatography; UV light absorption; fluorescence; nuclear magnetic resonance; and mass spectrometry.

[0040] Such endophytes may be characterised by application of microsatellite polymerase chain reaction amplification and product size analysis applied to DNA extracts of either endophyte *in planta;* endophyte; endophyte in a culture; and combinations thereof.

[0041] The combination of examples of a class of *N. lolii* endophyte and improved plant cultivars by artificial inoculation produces grass which do not cause symptoms of toxicosis by way of the ergovaline toxin but which contain indole diterpene compounds which continue to protect the host grass from pests or abiotic stresses (such as water deficit) or both.

[0042] Endophytes of the class of this disclosure may be characterised by examination of the properties of the endophytes in culture and in association with grass hosts.

[0043] The invention has been achieved by understanding the biology of endophytes of temperate climate grasses, isolating selected endophytes of interest, inoculating the endophytes Into surface-sterilised seedlings of grasses, re-evaluating alkaloid production, multiplying seed, evaluating for agronomic factors, testing for animal production, evaluating for any evidence of animal disorders such as ryegrass toxicosis, hyperthermia, or prolactin hormone depression.

[0044] The invention may be further described with reference to the appended claims.

BEST MODES FOR CARRYING OUT THE INVENTION

Culture conditions and description

[0045] The endophytes of this disclosure are strains from collections of seed of perennial ryegrass originally sourced from France. Seed from many various ryegrass collections from many countries were examined for the presence of endophyte by seed squash technique. A few plants for each seed sample, where endophyte was shown to be present, were grown for a few weeks in glasshouse conditions and re-tested for endophyte presence in their leaf sheaths.

[0046] The endophytes from plants with chemotypes of interest, primarily those not producing lolitrem B or ergovaline were isolated and grown in culture according the method of Latch and Christensen (1985). The endophytes of this invention are held in seed stocks, a culture collection, or in cloned plants at the AgResearch Ltd site in Palmerston North, New Zealand. The cultures are also deposited at the Australian Government Analytical laboratories in Sydney, Australia.

[0047] All strains of endophyte of interest can be accommodated within a single sub-grouping of the species *Neotyphodium lolii.* The isolates when grown on potato dextrose agar at 22° C are typically slow growing (radial growth approximately 0.1 - 0.3 mm per day) with colonies typically white and cottony, becoming fawn with age. Conidia have not been observed.

Inoculations

[0048] Axenic cultures of endophyte AR37 were successfully inoculated (Latch and Christensen, 1985) into seedlings grown from surfaced sterilised seed of perennial ryegrass cultivars *Lolium perenne,* for example Grasslands Nui and various experimental lines, generally with a satisfactory success rate usually in excess of 5% of attempts. Similarly annual ryegrasses *Lolium multiflorum,* for example Grasslands Moata, and Corvette, and hybrid ryegrasses *Lolium x hybridum* have been successfully inoculated for further examination with the chemotype characteristics of the combinations substantially the same as for perennial ryegrasses.

Chemotype identification

[0049] Basal parts of endophyte-infected tillers were freeze dried, sometimes milled, and extracted and analysed qualitatively for the presence or absence of peramine, lolitrems and ergovaline by high performance liquid chromatography (HPLC) using minor modifications of the methods of Barker et al., (1993) and Spiering et al., 2002. Some endophytes from such selections lacking both lolitrems and ergovaline were isolated, classified by culture attributes, and generally re-inoculated into seedlings of endophyte-free perennial ryegrass, cultivar Grasslands Nui, as a typical improved pasture host for comparative purposes. Samples from such plants at various stages of growth were analysed in more detail for alkaloid production. Following seed multiplication two groups of endophyte-grass combinations (with and without peramine in excess of 5 ppm) were tested in field plot trials to further determine their general agronomic qualities, persistence, and practical resistance to insect predation. Some endophytes, not of this invention, produce peramine but not lolitrems nor ergovaline and are the subject of USA Patent 6,072,107.

[0050] The endophytes of interest are of a class that does not produce lolitrem B (or other closely related lolitrems of similar chromatographic and fluorescence properties) or ergovaline at detection levels of 0.1 ppm of herbage dry matter. Neither do they normally produce peramine at a detection level of 1 ppm of herbage dry matter.

Identification of new alkaloids

[0051] The endophytes of interest produce indole diterpenes not seen before from any grass infected with endophytes. Typically 50 mg portions of ground freeze dried herbage of plants infected with these endophytes were extracted for 1 hour with 1 ml of dichloroethane-methanol 9:1 by volume, and the extract collected by centrifugation or filtration. The extracts were examined for the presence or absence of lolitrems by normal phase HPLC, for example with Alltima silica 150 x 4.6 mm columns (Alltech Associates, Deerfield, Il) and dichloromethane-acetonitrile, 7:1 by volume at 1 ml/min using fluorescence detection (excitation 265 nm, emission 440 nm). Two fluorescent peaks were observed with the endophytes of interest that are not characteristic of the *N. lolii* endophytes normally producing lolitrems. One of the peaks (A) was less retained than lolitrem B while another peak (B) was more retained. The same general pattern peaks was observed for extracts of herbage containing endophytes AR37 and AR40.

[0052] Extracts were also analysed by reverse phase HPLC, typically with a Prodigy 150 x 4.6 mm column (Phenomenex, Torrance, CA, USA) and with a solvent mixture of typically 5.6:1 (v/v) acetonitrile:aqueous ammonium acetate buffer (0.005 M) adjusted to pH 6 with acetic acid. The solvent flow rate was 1 ml/min, and eluted peaks were detected by fluorescence (excitation 265 nm, emission 440 nm or excitation 333 nm, emission 385). The order of elution was reversed and resolution enhanced in comparison to the above normal phase separation. The fluorescent peaks identified here as components I, II, III, and IV had retention times 7.7, 21.5, 24.2, and 25.1 min respectively for the above typical separation conditions. The normal phase peak B corresponded to reverse phase component I while the normal phase peak A resolved into three components II, III, and IV. The chemical identity of these components was further investigated.

[0053] UV and fluorescence spectra of components I, II, III, and IV were obtained by reverse phase HPLC using diode array and fluorescence stopped-flow techniques (Shimadzu SPD-M10A and RF-10A detectors) with spectral maxima as in Table 1. These data compare substantially to the spectra reported for the indole diterpene class of janthitrems (Gallagher, 1980; de Jesus *et al.,* 1984) or related shearinines (Belofsky, 1995).

Table 1: UV absorption and fluorescence spectral peaks

Component	UV	Fluorescence
	λ_{Max} nm	$\lambda_{Em\ Max}$ nm (λ_{Ex} 260 nm)
I	259, 333	381
II	259, 333	383
III	259, 333	387
IV	259, 333	384

[0054] HPLC with mass spectrometry (LC-MS) was performed using reverse phase chromatography with electrospray ionisation (ESI) (Shimadzu QP-8000α detector) and with variations of scan range and deflector voltage to induce and explore ion fragmentation. Table 2 lists the *m*/*z* of the indicated MH^+ ions together with major fragment ions. The loss of a fragment of mass 58 (assigned here as a loss of Me_2CO) has been reported for EI MS of janthitrem C (Penn *et al.,* 1993) and shearinine B (Belofsky, 1995).

Table 2: Mass spectral peaks from ESI LC-MS

Component	ESI mass spectral peak attributions				
	MH^+ *m*/*z*	MH^+ - H_2O	MH^+ -Me	MH^+ -Me_2CO	MH^+ -C_5H_9
I	646.5	628.4	-	588.3	-
II	670.5	-	655.1	612.4	600.95
III	672.5	-	-	614.6	-
IV	714.5	-	-	656.3	-

[0055] The further isolation and characterisation of component I was achieved by extracting 715 g of perennial ryegrass seed infected with endophyte AR37 with 3 litres of dichloromethane (DCM) at ambient temperature with stirring for 1.5 - 2 hr followed by a further 2 litres of DCM similarly treated. The combined extract was concentrated under reduced pressure and redissolved in hexane for a cycle of flash chromatography (Merck Silica Gel 60 0.040 - 0.063 mm, 170 g, 85 mm i.d.) with elution in 500 ml volume steps of hexane:DCM, DCM, DCM:acetonitrile (in proportions 19:1, 9:1, 4:1, and 1:1) and acetonitrile (MeCN). The fraction eluting with DCM:MeCN (4:1) was enriched with I and was evaporated to dryness (0.04 g), redissolved in a small volume of DCM:MeCN (4:1) and coated on to C-18 silica gel (2 g). This was put on top of a reverse phase silica gel flash column (Alltech octadecyl coated, 32 g, 28 mm i.d.) and fractions were eluted with 70 ml volumes of MeCN:H_2O in steps (1;1, 7:3, 4:1, 4:1, 9:1), MeCN, and DCM. The second MeCN:H_2O 4:1 fraction enriched in I was concentrated and used in two portions for flash chromatography on amino-coated silica (Analytichem Sepralyte Primary Secondary Amine, 2.1 g, 11 mm i.d.). Fractions were eluted with 5 ml volumes of MeCN:H_2O (1:1) and MeCN:H_2O (7:3). The MeCN:H_2O (1:1) fractions were concentrated to reduce volume, absorbed on a C-18 SPE column (2 g, 11 mm i.d.), eluted with MeCN and concentrated for examination by high resolution mass spectrometry and ^1H and ^{13}C NMR.

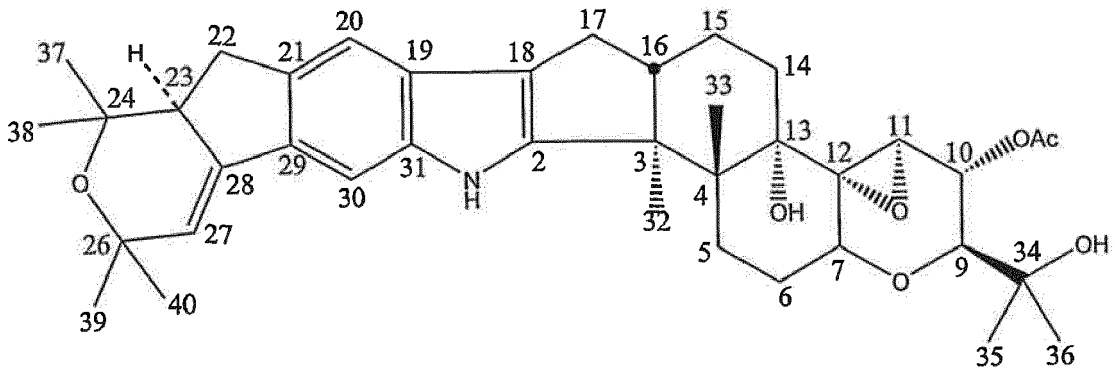
[0056] The high resolution mass spectrum obtained on a VG 70-250S mass spectrometer with a DCI probe yielded characterising ions with *m*/*z* 645.3647 (M^+) (calculated for $C_{39}H_{51}NO_7$: 645.3665) and *m*/*z* 630.3451 (M^+-Me) (calculated for $C_{38}H_{48}NO_7$: 630.3431).

[0057] Samples of I were examined in nuclear magnetic resonance (NMR) experiments to support a proposed structure of I which is also consistent with the high resolution masses.

[0058] NMR spectra were recorded in deuterioacetone ($(CD_3)_2CO$) solvent on a Bruker AC400 spectrometer. Chemical shifts are reported relative to TMS. The experiments included one-dimensional ^{13}C (100.62 MHz) and ^1H (400.13 MHz) spectra together with short-range and long range proton-proton (COSY) and proton-carbon correlation coupling (HMBC and HMQC) spectra. Signals were assigned by comparison with published NMR data for janthitrems (de Jesus *et al.,* 1984; Wilkins *et al.,* 1992; Penn *et al.,* 1993) and shearinines (Belofksy *et al.,* 1995), supported by the correlation data.

[0059] The proposed structure may be considered an epoxide of the known janthitrem G (de Jesus *et al,* 1984) and hence trivially named as 11,12-epoxy-janthitrem G (Figure 1).

[0060] The structure and numbering system for I is:



[0061] The supporting chemical shift data is in Table 3.

Table 3: NMR chemical shifts

Atom	^{13}C	^{1}H	Atom	^{13}C	^{1}H
2	154.3		23	49.3	2.82
3	51.1		24	74.2	
4	42.6		26	72.4	
5	26.4	1.62, 2.60	27	119.1	5.90
6	28.2	1.80, 2.21	28	140.8	
7	71.8	4.17	29	133.0	
9	76.0	3.45	30	103.5	7.36
10	68.2	5.14	31	140.4	
11	61.8	3.52	32	16.0	1.35
12	70.8		33	18.3	1.16
13	77.4		34	69.9	
14	29.7	1.58	35	26.1	1.13
15	21.0	1.50, 1.90	36	26.3	1.12
16	50.3	2.70	37	22.0	1.05
17	27.2	2.31,2.60	38	30.1	1.25
18	116.3		39	31.8	1.32
19	127.2		40	29.9	1.27
20	113.8	7.13	Acetate Me	20.8	2.09
21	136.4		Acetate CO	170.0	
22	32.0	2.63, 3.06			

[0062] By comparison and analysis of the UV, fluorescence and mass spectra we propose structures for II - IV:

II: The 10-deacetyl -10,34-(3-methylbut-2-enyl acetal) derivative of I.

III: The 10-deacetyl-34-O-(3-methylbut-2-enyl) derivative of I.

IV: The 34-O-(3-methylbut-2-enyl) derivative of I.

Genotype characterisation of endophyte

[0063] All endophytes so far tested are characterised by DNA "fingerprinting" (selected polymorphic microsatellite loci and/or AFLP technique) as belonging to a sub-group of *Neotyphodium lolii.*

[0064] Samples of about 50 mg fresh or 15 mg dry basal tiller were used for the extraction of DNA using FastDNA kit for plants (Bio 101, Vista, California) using procedures recommended with the kit. Alternatively genomic DNA was extracted from cultured endophyte (Moon et al., 1999). Microsatellite PCR amplification was performed using primer pairs labelled with fluorescent dyes, B10.1 (5'-TET) / B10.2 and B11.1 (5'-HEX) /B11.4, as described by Moon et al.,

(1999). The apparent size of microsatellite PCR fluorescent-labelled products was measured relatively to within an estimated 0.3 nucleotide units by capillary electrophoresis using an ABI 3100 Genetic Analyzer with POP6 polymer chemistry in 50 cm capillary arrays and GeneScan-400HD standards (Applied Biosystems Inc., Foster City, CA).

[0065] The apparent sizes of PCR products by this technique (adjusted by subtracting a unit where an adenine nucleotide appears to have been terminally added) are in Table 4 and show that the endophytes of interest may be distinguished from other groups of *N. lolii* endophytes by the apparent sizes of alleles. Thus the strains may be characterised by B10 allele of apparent size about 160.6 and a B11 allele of apparent size about 132.0. Other strains of *N. lolii* and some of *Epichloë festucae* have been shown to generally have a single B10 allele with apparent size about 175.6 and a single variable apparent sized B11 allele although the size 132.0 was not observed in any endophyte outside the endophytes of interest . A single allele for each locus is typical of *N. lolii* and *Epichloë festucae.*

Table 4. Apparent size of B10 and B11 microsatellite PCR products

Source material	B10 allele size	B11 allele size
N. lolii strain Lp19	175.7	180.3
N. lolii strain Lp7	175.6	188.3
AR29 (*N. lolii* strain from Grasslands Nui ryegrass)	175.7	176.2
AR5 (a strain lacking lolitrem B)	175.6	240.7
AR1 (a strain lacking both lolitrem B and ergovaline)	175.7	147.8
FI1 (*Epichloë festucae* from *Festuca longifolia*)	175.6	115.6
AR37	160.6	132.0
AR40	160.7	132.0

[0066] The finding of single sizes of alleles (B10 = c. 160.6 and B11 = c. 132.0) for endophytes of interest does not preclude a possibility that closely related endophytes with the same functional properties might have different alleles.

[0067] Analysis by AFLP (Griffiths et al., 1999) also confirmed that endophyte examples AR37 and AR40 are from a sub-group that can be distinguished from other *N. lolii* endophytes outside this sub-group by one or more polymorphic differences from within more than 200 AFLP bands observed to be polymorphic for the genus *Neotyphodium.*

Endophyte and growth of pasture

[0068] The growth of the cultivar Grasslands Nui infected with AR37 and wild-type, and endophyte-free was assessed in a series of field trials, both grazed and mown, in four regions of New Zealand over a period of more than 3 years from 1996.

[0069] Plots infected with AR37 generally yielded more ryegrass herbage than wild-type plots. In 11 trials sown in 1996 and 1997 annual yields measured from AR37 plots were on average 11% greater over 3 years. The greatest differences occurred from late summer through autumn.

[0070] For example, in Site 1, where conditions are favourable for good ryegrass growth (e.g. wild-type yields 15000 kg DM/ha/year), AR37 plots yielded 6% more annual herbage ($P<0.05$) with the greatest yield advantages in the autumn (Table 5). At another site, Site 2, less favourable for ryegrass growth and persistence (e.g. wild-type yields 8700 kg DM/ha/year), AR37 plots had higher yields in all seasons and significantly so for 3 seasons and for total annual yields (Table 5).

Table 5. Ryegrass yields of Grasslands Nui infected with AR37 relative to yields of Nui with wild-type endophyte (=100) for field plots at two contrasting locations. Average of yields for 3 years for trials sown in autumn 1996

Site	Winter	Spring	Summer	Autumn	Annual
Site 1	108	100	107	120 *	106 *
Site 2	113	114 *	117 *	123 *	116 *
* Indicates value at the site is significantly different to wild-type ($P < 0.05$)					

[0071] At Site 2, another trial sown in 1998 with Nui ryegrass and a ryegrass selection known as 'GA66' resulted in higher annual yields for AR37 plots for both ryegrasses (+15% and +14%) ($P<0.05$) compared with wild-type plots.

[0072] Differences in number of tillers were apparent from mid-summer to early winter, being from 22% to 64% greater for AR37 compared with wild-type (P<0.05) (Table 6).

Table 6. Grasslands Nui ryegrass tiller numbers in autumn (per metre row at Site 3, per m^2 at site 1)

Site	AR37	Wild-type	Endophyte-free
Site 3, Area 1	1340 [a]	1100 [b]	1120 [b]
Site 3, Area 2	1680 [a]	1300 [b]	1030 [b]
Site 1	7200 [a]	4400 [b]	4100 [b]

[0073] For each site numbers without a letter in common are significantly different (P < 0.05)

[0074] Total root organic matter was examined in a trial at Site 3 after Grasslands Nui rows were occasionally mown to simulate rotational grazing. Cores, 25 mm diameter by 300 mm soil depth were assessed and the grass infected with AR37 shown to have significantly more root mass than either endophyte-free or wild-type infected grass (Table 7).

Table 7. Root mass (grams organic dry matter per core)

	AR37	Wild-type	Endophyte-free
Total root organic matter	2.05 [a]	1.39 [b]	1.42 [b]

[0075] Numbers without a letter in common are significantly different (P < 0.05)

[0076] Thus it was shown that infection of perennial ryegrass cultivars with AR37 results in generally superior pasture growth and potential pasture productivity especially in late summer and autumn.

Endophyte and growth of turf

[0077] Perennial ryegrass is frequently used as a main component of utility turf for aesthetic and recreational purposes. An observation that Grasslands Nui cultivar infected with AR37 had persistence and green colour compared to other endophyte infections of Grasslands Nui during a dry summer season in a further site, Site 4, stimulated a small plot trial comparison of Grasslands Nui infected with either its own natural high level of wild-type endophyte or artificially infected with AR37. Trials were conducted at Site 4 and at Site 1.

[0078] The plots were managed to simulate turf growth conditions and typical turf management with regular mowing to 2 cm height when the height had grown to an estimated 3 cm. Fertiliser was applied at 30 units of nitrogen per month generally when raining and discontinued during drought periods. Water was applied only to avoid plant death from desiccation.

[0079] The measurements made included tiller density, grass production (mowing), observations on disease and pests, soil moisture and bulk density, root mass and top mass (under the mower height) and plant morphology measurements including leaf and sheath dimensions.

[0080] Although there was little difference in yield above mower height there were differences in grass mass below mower height, particularly at Site 1 where the AR37 plot was about double the wild-type treatment (P<0.001).

[0081] The tiller density per unit of area at both Site 1 and Site 4 was significantly greater for AR37 plots (P<0.005). Similarly root mass was consistently higher with AR37 plots by about 25% or more (P<0.02) at both sites. Leaf (P<0.03) and sheath (P<0.02) widths, measured at the base of each part, were consistently less for AR37 plots measured just at Site 4. The mean tiller dry matter for AR37 was approximately 40% less than for wild-type (P<0.014) at Site 4 however the mean number of leaves per tiller was very nearly three for both endophyte plots and not significantly different.

[0082] Thus it was shown that infection with AR37 of Grasslands Nui results in a denser sward of smaller tillers when managed as a turf. These swards have increased root mass and herbage below cutting height compared to wild-type endophyte. These characteristics have high utility for improving the ground cover and lateral shear strength of turf systems.

Endophyte and pest protection

[0083] The endophytes used in this invention provide their host perennial ryegrass with resistance to a range of insect pests including Argentine stem weevil, black beetle, mealy bug and root aphid. In a combination of field and pot trials the degree of protection provided by the AR37 endophyte when compared with endophyte-free ryegrass is equivalent to that provided by the naturally occurring wild-type endophyte for all these pests except root aphid against which the wild-type endophyte provides little or no protection (Table 8).

[0084] For Argentine stem weevil (*Listronotus bonariensis*) the mode of resistance afforded by endophyte differs between AR37 and the wild-type. In AR37 adult feeding and oviposition are the same as in endophyte-free plants whereas in the wild-type defence against the weevil is mediated primarily via deterrence of the adult from feeding and oviposition by the alkaloid peramine. Observations indicate that AR37 reduces larval damage to tillers because it is toxic to larvae, AR37 has been tested against Argentine stem weevil extensively in field and in pot trials and has consistently reduced damage by this pest to low levels when compared to damage in endophyte-free ryegrass.

[0085] AR37 also reduces black beetle (*Heteronychus arator*) damage by larvae in the field, mainly through deterrence of the adult. Adult black beetle damage to ryegrass tillers infected with AR37 was 17.3% whereas 46% of endophyte-free tillers were damaged. Survival of root aphid (*Aploneura lentisci*), mealy bug (*Balanococcus poae*) and porina (*Wiseana cervinata*) are also less on ryegrass with AR37 than on endophyte-free ryegrass.

Table 8. Examples of the effect of AR37 on different insect pests

Insect	Parameter	AR37	Wild-type	Endophyte-free
Argentine stem weevil	% Tillers with larval damage	13^{a}	17^{a}	36^{b}
Black beetle	No. larvae/m^2	13.8^{a}	13.8^{a}	60.0^{b}
Root aphid	Log (n + 1)/plant	0.27^{a}	1.61^{b}	2.13^{b}
Mealy bug	No./10 cores	0.3^{a}	0.6^{a}	16.8^{b}
Porina	% survival	50.2^{a}	60.0^{ab}	89.5^{b}

[0086] For each insect, numbers without a letter in common are significantly different ($P < 0.05$)

Endophyte and animal performance

[0087] Sheep grazing ryegrass cultivars with their wild-type endophyte in summer and autumn may exhibit one or all of the symptoms of ryegrass-endophyte toxicosis. These include reduced live weight gain, ryegrass staggers, increased rectal temperatures and respiration rates, especially in warm humid conditions, increased incidence of faecal soiling (dags) and fly strike and reduced basal prolactin levels. Using these parameters, the health and production responses of sheep grazing the same ryegrass cultivar without endophyte, with its wild-type endophyte or with AR37 endophyte in summer and autumn over 3 years were compared (Table 9).

Table 9: Mean responses (3 years) of sheep grazing ryegrass with AR37 compared to same ryegrass without endophyte or with its wild-type endophyte

	Endophyte-free	Wild-type	AR37
Live weight change (g/day)	62	-12	47
Ryegrass staggers (0-5 ascending scale)	0	2.7	1.8
Rectal temperature (°C)	40.4	40.7	40.5
Respiration rate (breaths/minute)	85	109	95
Plasma prolactin (ng/ml)	208	110	210

[0088] The sheep grazing endophyte-free ryegrass exhibited none of the adverse responses typically associated with ryegrass-endophyte toxicosis. Those grazing ryegrass with AR37 had mild ryegrass staggers but the incidence and severity was significantly less than for those sheep grazing ryegrass with its wild-type endophyte. Mean live weight change was slightly lower than for those grazing endophyte-free but significantly better than the negative growth rates of those grazing ryegrass with wild-type endophyte. For all the other parameters (rectal temperature, respiration rate and plasma prolactin levels) measured there was no significant difference between sheep grazing endophyte-free ryegrass and those grazing ryegrass with AR37. However respiration rates and rectal temperatures were significantly higher for sheep grazing ryegrass with its wild-type endophyte than for those grazing AR37, while plasma prolactin levels were significantly lower for ryegrass with wild-type endophytes.

[0089] In another replicated trial there was no evidence of ryegrass staggers in sheep grazing endophyte-free ryegrass cultivars with AR37 whereas on the same ryegrass cultivars with wild-type endophyte the sheep had serious ryegrass

staggers. Mean live weight gains in sheep grazing AR37 treatments were 130 g/day whereas those grazing the same ryegrass with its wild-type endophyte grew at only 90 g/day.

[0090] In a larger on-farm grazing trial where the ryegrass was sown with clover, responses were similar in sheep grazing AR37 treatments to those on endophyte-free treatments with no ryegrass staggers on AR37 treatments.

[0091] Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof as defined in the appended claims.

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Claims

1. A method of protecting a host grass from biotic and abiotic stresses by artificially inoculating the host grass with a strain of endophyte of *Neotyphodium lolii* species, **characterized in that** the strain of endophyte of *N. lolii* produces 11,12-epoxy-janthitrem G at a level sufficient to confer said protection to the host grass.

2. The method as claimed in claim 1, wherein the artificially inoculated host grass has enhanced root growth and more tillers in comparison to a grass without endophyte infection.

3. The method as claimed in claim 1 or claim 2 wherein the biotic stresses are caused by pests and insects.

4. The method as claimed in claim 3 wherein the pests to which increased resistance is conferred on the host grass are selected from the group consisting of: root aphid (*Aploneura lentisci*); mealy bug (*Balanococcus poae*); black beetle (*Heteronychus arator*); porina (*Wiseana cervinata*); and combinations thereof.

5. The method as claimed in any of the above claims wherein the host grass is a Pooideae grass and wherein the

endophyte strain confers the features of enhancement of grazing animal growth and increased animal productivity in comparison with grass infected with endophytes known to induce ryegrass-endophyte toxicosis.

6. The method as claimed in any of the above claims wherein the abiotic stress is a water deficit.

7. The method as claimed in any of claims 1-4 or 6 wherein the host grass is a perennial, annual or hybrid ryegrass, preferably wherein the host grass is a Pooideae grass, preferably wherein the host grass is selected from the species: *Lolium perenne; Lolium multiflorum;* and *Lolium x hybridum.*

8. The method as claimed in any of the above claims wherein the endophyte strain also does not produce sufficient levels of a compound or compounds to cause toxicosis in grazing animals, preferably wherein the toxicosis is ryegrass-endophyte toxicosis.

9. The method of claim 8, wherein the toxicosis avoided is caused by: ergovaline toxin, lolitrem toxin, and a combination thereof.

10. The method as claimed in any of the above claims wherein the endophyte strain produces toxic alkaloids lolitrem B and ergovaline at levels of less than 2 ppm of dry matter lolitrem B and 0.5 ppm of dry matter ergovaline, preferably wherein the endophyte strain produces toxic alkaloids lolitrem B and ergovaline at detection levels of less than 0.1 ppm of dry matter.

11. The method as claimed in any of the above claims, wherein the *Neotyphodium lolii* endophyte strains have base pair allele sizes of 160.6 at the B10 allele and 132.0 at the B11 allele.

12. The method of any of the above claims wherein the endophyte strain is in the form of an endophyte culture.

13. The method as claimed in any of the above claims wherein infection is achieved by modifying the host grass using techniques selected from the group consisting of: breeding, crossing, hybridisation, selection and genetic modification.

14. Use of a strain of endophyte of *N. lolii* species that produces 11,12-epoxy-janthitrem G. in the protection of a host grass from biotic and abiotic stresses, the strain of endophyte of *N. lolii* species conferring increased abiotic and biotic protection when artificially inoculated into said host grass.

Patentansprüche

1. Verfahren zum Schützen eines Wirtsgrases vor biotischen und abiotischen Belastungen durch künstliches Beimpfen des Wirtsgrases mit einem Endophytenstamm der Spezies *Neotyphodium lolii,* **dadurch gekennzeichnet, dass** der Endophytenstamm von *N. lolii* 11,12-Epoxy-janthitrem G. in einer Menge erzeugt, die ausreicht, um dem Wirtsgras den Schutz zu verleihen.

2. Verfahren nach Anspruch 1, wobei das künstlich beimpfte Wirtsgras im Vergleich zu einem Gras ohne Endophyteninfektion ein verstärktes Wurzelwachstum und mehr Schösslinge aufweist.

3. Verfahren nach Anspruch 1 oder 2, wobei die biotischen Belastungen durch Schädlinge und Insekten bewirkt werden.

4. Verfahren nach Anspruch 3, wobei die Schädlinge, gegen die dem Wirtsgras ein verstärkter Widerstand verliehen wird, ausgewählt sind aus der Gruppe bestehend aus: Galllaus (*Aploneura lentisci*); Schmierlaus (*Balanococcus poae*); Schwarzem Käfer (*Heteronychus arator*); Porina (*Wiseana cervinata*); sowie Kombinationen daraus.

5. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Wirtsgras ein Pooideae-Gras ist und wobei der Endophytenstamm die Merkmale der Verstärkung von Weidetierwachstum und erhöhter Tierproduktivität im Vergleich zu Gras verleiht, welches mit Endophyten infiziert ist, von denen bekannt ist, dass sie Lolchendophyten-Toxikose induzieren.

6. Verfahren nach einem der vorhergehenden Ansprüche, wobei die abiotische Belastung ein Wassermangel ist.

7. Verfahren nach einem der Ansprüche 1-4 oder 6, wobei das Wirtsgras ein mehrjähriger, einjähriger oder Hybrid-Lolch ist, vorzugsweise wobei das Wirtsgras ein Pooideae-Gras ist, vorzugsweise wobei das Wirtsgras ausgewählt ist aus den Spezies: *Lolium perenne; Lolium multiflorum;* und *Lolium x hybridum.*

8. Verfahren nach einem der vorhergehenden Ansprüche, wobei der Endophytenstamm ebenfalls keine ausreichenden Mengen einer Verbindung oder von Verbindungen erzeugt, um Toxikose in Weidetieren zu verursachen, vorzugsweise wobei die Toxikose Lolchendophyten-Toxikose ist.

9. Verfahren nach Anspruch 8, wobei die vermiedene Toxikose verursacht wird durch: Ergovalin-Toxin, Lolitrem-Toxin und eine Kombination daraus.

10. Verfahren nach einem der vorhergehenden Ansprüche, wobei der Endophytenstamm toxische Alkaloide Lolitrem B und Ergovalin in Mengen von weniger als 2 ppm Trockenmasse Lolitrem B sowie 0,5 ppm Trockenmasse Ergovalin erzeugt, vorzugsweise wobei der Endophytenstamm toxische Alkaloide Lolitrem B und Ergovalin mit Erfassungsgehalten von weniger als 0,1 ppm Trockenmasse erzeugt.

11. Verfahren nach einem der vorhergehenden Ansprüche, wobei die *Neotyphodium lolii*-Endophytenstämme Basenpaar-Allelgrößen von 160,6 am B10-Allel sowie 132,0 am B11-Allel aufweisen.

12. Verfahren nach einem der vorhergehenden Ansprüche, wobei der Endophytenstamm in der Form einer Endophytenkultur vorliegt.

13. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Infizieren durch Modifizieren des Wirtsgrases unter Einsatz von Techniken erzielt wird, die ausgewählt sind aus der Gruppe bestehend aus: Züchten, Kreuzen, Hybridisieren, Selektion und genetischer Veränderung.

14. Gebrauch eines Endophytenstammes der Spezies *N. lolii,* der 11,12-Epoxy-janthitrem G. unter dem Schutz eines Wirtsgrases vor biotischen und abiotischen Belastungen erzeugt, wobei der Endophytenstamm der Spezies *N. lolii* erhöhten abiotischen und biotischen Schutz verleiht, wenn künstlich in das Wirtsgras eingeimpft.

Revendications

1. Procédé de protection d'une herbe hôte contre des contraintes biotiques et abiotiques en inoculant artificiellement l'herbe hôte avec une souche d'endophytes de l'espèce *Neotyphodium lolli,* **caractérisé en ce que** la souche d'endophytes de *N. lolli* produit le 11,12-epoxy-janthitrem G à un taux suffisant pour conférer ladite protection à l'herbe hôte.

2. Procédé selon la revendication 1, dans lequel l'herbe hôte inoculée artificiellement a une croissance de racines renforcée et plus de talles comparée à une herbe sans infection d'endophytes.

3. Procédé selon la revendication 1 ou la revendication 2 dans lequel les contraintes biotiques sont causées par des animaux nuisibles et des insectes.

4. Procédé selon la revendication 3 dans lequel les animaux nuisibles contre lesquels une résistance renforcée est conférée à l'herbe hôte sont sélectionnés parmi le groupe constitué de : l'aphide des racines (*Aploneura lentisci*) ; la cochenille (*Balanococcus poae*) ; le coléoptère (*Heteronychus arator*) ; la chenille (*Wiseana cervinata*) ; et des combinaisons de ceux-ci.

5. Procédé selon l'une quelconque des revendications précédentes dans lequel l'herbe hôte est une herbe Pooideae et dans lequel la souche d'endophytes confère les caractéristiques de renforcement de la croissance d'animaux au pâturage et de productivité augmentée des animaux comparée à l'herbe infectée par des endophytes connus pour induire une toxicose à endophytes de ray-grass.

6. Procédé selon l'une quelconque des revendications précédentes dans lequel la contrainte abiotique est un déficit en eau.

7. Procédé selon l'une quelconque des revendications 1 à 4 ou 6 dans lequel l'herbe hôte est du ray-grass vivace,

annuel ou hybride, de préférence dans lequel l'herbe hôte est une herbe Pooideae, de préférence dans lequel l'herbe hôte est sélectionnée parmi les espèces : *Lolium perenne ; Lolium multiflorum ;* et *Lolium x hybridum.*

8. Procédé selon l'une quelconque des revendications précédentes dans lequel la souche d'endophytes ne produit pas non plus des taux suffisants d'un composé ou de composés pour causer une toxicose chez les animaux au pâturage, de préférence dans lequel la toxicose est une toxicose à endophytes de ray-grass.

9. Procédé selon la revendication 8, dans lequel la toxicose évitée est causée par : la toxine ergovaline, la toxine lolitrem, ou une combinaison de celles-ci.

10. Procédé selon l'une quelconque des revendications précédentes dans lequel la souche d'endophytes produit du lolitrem B et de l'ergovaline alcaloïdes toxiques à des taux inférieurs à 2 ppm de lolitrem B sur une base de matière sèche et de 0,5 ppm d'ergovaline sur une base de matière sèche, de préférence dans lequel la souche d'endophytes produit du lolitrem B et de l'ergovaline alcaloïdes toxiques à des taux de détection inférieurs à 0,1 ppm de matière sèche.

11. Procédé selon l'une quelconque des revendications précédentes, dans lequel les souches d'endophytes *Neotyphodium lolii* ont des tailles d'allèles de 160,6 paires de bases à l'allèle B10 et de 132,0 paires de bases à l'allèle B11.

12. Procédé selon l'une quelconque des revendications précédentes dans lequel la souche d'endophytes est sous forme d'une culture d'endophytes.

13. Procédé selon l'une quelconque des revendications précédentes dans lequel l'infection est réalisée en modifiant l'herbe hôte par des techniques sélectionnées parmi le groupe constitué de : la culture, le croisement, l'hybridation, la sélection et la modification génétique.

14. Utilisation d'une souche d'endophytes de l'espèce *N. lolii* qui produit le 11,12-epoxy-janthitrem G pour conférer la protection d'une herbe hôte contre des contraintes biotiques et abiotiques, la souche d'endophytes de l'espèce *N. lolii* conférant une protection abiotique et biotique renforcée lorsqu'elle est inoculée artificiellement dans ladite herbe hôte.

REFERENCES CITED IN THE DESCRIPTION

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