Impact analysis of endophytes on the phenotype of varieties of *Lolium perenne* and *Festuca arundinacea*

Final Report of the examination Team, February 2016:

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1. Background

During the revision of the CPVO technical protocol for *Lolium* ssp. and *Festuca* ssp. at the agricultural experts meeting in 2010, it was questioned whether the presence of endophyte in varieties of perennial ryegrass *Lolium perenne* L (Lp) and *Festuca arundinacea* Schreb *(Fa) would* have an impact on their phenotypic Characteristics. Two species of endophyte are known to infect perennial ryegrass (*Neotyphodium lolii* and *N. occultans* (previously *Acremonium lolli*. & *A.occultans*) and the single infecting species in tall fescue is *N. Coenophialum*. There is no breeding of endophytes as it has not been possible to stimulate reproduction *in vitro*, but screening of populations have allowed the isolation and clonal multiplying of variants with differing properties, including presence/absence or reduced efficacy of insect and animal toxicity. Some of these variants are patent protected.

If the presence of an endophyte modifies grass plant morphology, this could potentially create a difference in the CPVO characters used to assess Distinctness, Uniformity and Stability (DUS) and result in an apparent distinctness between two accessions of the same variety, one with and the other without an endophtye inoculation.

It was accepted by the agricultural experts that to avoid this risk it would be necessary to impose a rule that required all seed submissions for Plant Breeders Rights (PBR)to be endophyte free. However, such a requirement would incur a costly cleaning procedure for the applicants, which the breeders' representatives declared as unacceptable to them. It was therefore agreed by the EU Community Plant Varieties Office (CPVO) and the European Seed Association (ESA) to co-fund a research study that would provide evidence of whether endophyte presence modified grass morphology.

In December 2011 a working group comprising CPVO, ESA and agricultural experts from the United Kingdom, France and Germany met and agreed the terms of reference for the project, its duration and the costs involved.

2. Description of the project

The plant material assessed comprised of four varieties of perennial ryegrass and four of tall fescue derived from a wide genetic/geographic base. Each variety was represented by two accessions, one containing endophyte (E-) and the other without endophyte (E+). Two breeding companies, Barenbrug and DLF Trifolium agreed to supply this plant material. This involved treating a aliquot of seed of each test variety to remove the endophyte. They grew individual plants of the resulting E- and E+ accessions in multipots and tested for endophyte presence status before delivering enough plants to produce a 60-plant DUS trial at each Examination Office (EO) that comprised of E- (0%

endophyte) and E+ (100% endophyte) accessions. This process was conducted twice to provide a different set of plants for sowing in 2013 (examined 2014) and in 2014 (examined 2015).

In addition, it was agreed among the three EO's to include their definitive sample of each test variety as part of the experiment, so providing an additional comparator to the experimentation. These definitive samples were assumed to be endophyte free as this is an existing condition for seed submitted for testing by these authorities. These additional accessions were not part of the CPVO/ESA funded study.

The perennial ryegrasses were examined by the Agri-Food and Biosciences Institute (AFBI) at Crossnacreevy, Northern Ireland, UK and the Federal Plant Variety Office, Bundessortenamt, Scharnhorst Germany. The tall fescue was tested by Geves, L'Anjouère, France. As the ryegrasses were tested by the UK and German EO's, the breeders divided every ryegrass plant in two and so sent an identical set of plants to each of the two EO's.

All the test accessions were integrated into the regular DUS trials at each EO and examined for the CPVO permitted DUS characters in compliance with the relevant CPVO-Technical Protocol during the two growing cycles. Further specific details of the experimental set-up are provided in Annex 1.

3. Results

An interim report was presented to the CPVO Agricultural Technical Experts meeting in 2014, which detailed the findings of the first year trials. The current report summarises the findings of both the 2014 and 2015 years.

The complete experimentation generated:

48,960 observations for perennial ryegrass
2 locations x 2 growing cycles x 60 plants/accession = 240
x 4 varieties (Lol A, Lol B, Lol C, Lol D)
x 3 accessions = 2880 plants x max of 17 characters

12,960 observations for tall fescue
1 location x 2 growing cycles x 60 plants/accession = 120
x 4 varieties (Fes A, Fes B, Fes C, Fes D)
x 3 accessions = 2880 plants x max of 9 characters

This combination of four varieties by three accessions in each species, produced a total of 132 pairwise comparisons (4var x 3 accessions x 11 pairs), giving an overall total across all three test sites and both species of 396. Therefore it is not practical to provide tables of all of these extensive data sets. However, this proved unnecessary as the same responses to 'with' and 'without' endophyte was Fes Dly the same regardless of which species or location was studied.

<u>Occurrence of Significant Differences between E+ and E- Accessions</u>

When the combined over-years analyses for distinctness (COY-D) was performed all EO's some significant differences were observed between the E+ and E- accessions of several of the varieties. For example in Table 1, Lol B E+ and Lol B E- were found to be significantly different ear length and length of the basal spiklet at the 5% level of significance.

Table 1. Example comparison between two ryegrass varieties, with/without endophyte at AFBI Crossnacreevy

Comparison between Lol B E+ and Lol B E- following 2 years of testing T values positive if Lol B E+ larger than Carnius E-							
Character		**Mjra Analysis**			**F3**	**Significance**	
	Stringency	**T**	**Prob**	Signif.		2014	2015
4 ANG YOS	1.12	-0.46	64.277	NS	0.2S	-	-
70 SP.WDTH	1.03	0.24	81.385	NS	0.1	+	-
9 SP. ANG	0.98	-1.01	31.196	NS	1.8	-	+
5 SP.HGHT	0.95	-0.61	54.291	NS	0.8	-	+
8 DATE EE	0.96	-0.07	94.201	NS	0.2	+	-
10 HGHT EE	0.94	-0.21	83.236	NS	1.7	-	+
50 PlntShpe	1.38	-0.54	58.686	NS	1.1	+	-5
14 LGTH FL	0.95	-0.56	57.587	NS	0.0	-	-
15 WIDTH FL	0.99	0.89	37.254	NS	0.3	+	+
40 LeafShpe	0.89	-1.24	21.615	NS	0.3	-	-
17 LLSEE+30	0.98	-1.71	8.931	NS	0.1	-1	-
20 INT LGTH	0.92	-0.49	62.178	NS	0.3	+	-
24 EAR LGTH	0.98	-2.38	1.814	*-	0.0	-1	-1
31 SPKLT NO	1.04	-1.66	9.801	NS	0.2	-	-1
41 SpkDensity	0.98	-0.12	90.524	NS	0.1	-	+
34 GLUMLGTH	0.97	-1.91	5.75	NS	0.4	-2	-
35 LGHBSP-A	0.92	-2	4.652	*	0.7	-	-

For the same comparison conducted at Scharnorst there was also a significant difference between the two accessions at the 5% confidence limit (Table 2). In this case, however in this case the difference was in Plant Width, which was not observed at Crossnacreevy not where the two character differences found at Crossnacreevy were not repeated at Sharnhorst.

Table 2. Example comparison between two ryegrass varieties, with/without endophyte at Sharnhorst

Comparison between Lol B E+ and Lol B E- following 2 years of testing									
Code No	**Characteristic**	**Mean value**	**Mean value**	**Mean diff.**	**T**	**PROB**	**SIG**	**1%**	**5%**
M101	Flag leaf: length	141.13	142.77	-1.64	-0.15	88.424	NS	38.01	25.68
M102	Flag leaf: width	3.66	3.72	-0.06	-0.29	78.179	NS	0.83	0.55
M105	Flag leaf: length/width ratio	39.22	38.67	0.55	0.37	72.634	NS	5.61	3.7
M201	Plant: length of longest stem, + inflorescence	60.01	59.7	0.31	0.19	85.588	NS	6.06	4
M202	Inflorescence: length	17.12	17.53	-0.41	-0.62	55.976	NS	2.43	1.61
M205	Plant: length of upper internode	20.33	20.28	0.05	0.04	96.933	NS	4.39	2.97
M206	Inflorescence: number of spikelets	19.07	19.59	-0.52	-2.42	5.192	NS	0.8	0.53
M207	Inflorescence: density	89.93	89.85	0.09	0.05	96	NS	6.03	3.98
M208	Inflorescence: length of outer glume on basal spikelet	7.7	7.42	0.27	0.44	67.502	NS	2.2	1.49
M209	Inflorescence: length of basal spikelet excluding awn	11.54	11.81	-0.27	-0.22	83.294	NS	4.32	2.92
M301	Plant: time of inflorescence emergence (after vernalization)	53.77	52.89	0.88	0.96	36.981	NS	3.2	2.16
M401	Plant: natural height at inflorescence emergence	45.53	44.92	0.61	0.34	74.321	NS	6.21	4.2
M402	Plant: growth habit at inflorescence emergence	5.22	5.14	0.08	0.7	50.855	NS	0.42	0.28
M502	Plant: vegetative growth habit (without vernalization)	6.58	6.44	0.14	1.01	34.763	NS	0.49	0.33
M506	Plant: width (after vernalization)	36.19	35.25	0.94	2.42	4.591	*	1.36	0.92
Note naming of characters differs between Crossnacreevy and Sharnhorst									

Example from, Fes C E+ compared to E-

Similar to the ryegrass analyses, Geves also found significant differences between some E+ and E- accessions of the tall fescue varieties. Table 3 shows an example where, after two years of testing at L'Anjouère, there were no significant differences.

Table 3. Example comparison between two tall fescue varieties, with/without endophyte at L'Anjouère

Comparison between Fes C E+ and Fes C E- following 2 years of testing														
	2 by 1% Method					**COY-D Analysis**								
	T Score		**Sig Level**			**Mean Differences**						**COY-D**		
Code No	**2013**	**2014**	**2013**	**2014**	**Sig**	**2013**	**2014**	**Mean**	**AJ. Mean**	**Est**	**T**	**Prob %**	**Sig nif**	**1%**
7	-1.74	0.13	-	+	N	-2.67	0.17	-1.25	-1.25	.	-0.75	45.38	NS	4.34
11*	0.33	-0.49	+	-	N	1.03	-1.58	-0.27	-0.27	.	-0.09	92.817	NS	7.93
12	-0.23	1.01	-	+	N	-0.06	0.36	0.15	0.18	.	0.78	43.743	NS	0.59
14*	-1.72	0.66	-	+	N	-11.35	6.16	-2.59	-0.44	.	-0.05	95.728	NS	21.26
13	-0.87	-0.57	-	-	N	-7.68	-5.73	-6.7	-6.7	.	-0.87	38.582	NS	20.11
B	0.82	-1.17	+	-	N	16.91	-25.93	-4.51	-4.51	.	-0.22	82.962	NS	54.62
10	-1.54	-0.11	-	-	N	-4.02	-0.28	-2.15	-2.05	.	-0.82	41.128	NS	6.48
8*	2.34	-0.65	2	-	N	2.73	-0.81	0.96	0.96	.	-0.79	43.336	NS	3.18
a	-1.77	-0.2	-	-	N	-2.45	-0.25	-1.35	-1.12	.	-1.19	23.52	NS	2.45
All characters were CPVO approved and codes relate to those used by Geves														

The critical question needing to be answered was whether these individual significant differences indicated a morphological change as a consequence of having an endophyte present or not. In order to do this it is necessary to understand the number of significant differences, the levels of significance and the characters in which they occurred. In Table 4 the high number of pair-wise comparisons that generated a significant difference at the $p<0.01$ probability are presented for the trials conducted at the Sharnhorst location. It can be seen that out of the four comparisons between E+ and E- accessions, three would have been declared as non-distinct comparisons in standard PBR trials.. These were the comparisons between Lol A E+/E-, Lol C E+/E- (both ns) and Lol B E+/E- (difference only at the $p<0.05$ level, which is below the distinctness threshold). The only one of the four variety accession comparisons for which a significant difference between with/without endophyte was observed was for Lol D E+/E-.

It is also notable that all of the 'between variety' comparisons were significant at the $p<0.01$ level, confirming that the DUS trial was successfully discriminating between these four varieties. Furthermore this also shows that the significant difference observed between Lol D E+/E- was equivalent to that expected to occur between truly different varieties. Had this study been performed as blind study, in which the identity of the varieties and their accessions had not been revealed to the EO's, then the comparison between Lol D accessions would have been declared as two distinct varieties. Similar comparisons were also reported at Crossnacreevy for the same varieties, where the two Lol D accessions were again significantly different at $p<0.01$. Furthermore the Crossnacreevy test centre also observed significant differences between Lol C E+/E- and between Lol B E+/E-. Only the Lol A E+/E- comparison showed no significant difference and would have resulted in its with and without endophyte accession being declared not distinct. However, at this test centre, the with and without endophyte accessions would have been declared as distinct varieties in a standard DUS test.

In contrast to the ryegrass results, none of the tall fescue variety accessions were found to be distinct. Each of the pair-wise comparisons between Fes A E+/E-, Fes C E+/E- and Fes D E+/E- had no significant differences at all and for the Fes B E+/E- comparison there was only a single difference at the $p<0.05$. As this is below the distinctness threshold, had a blind study been conducted at L'Anjouère, none of the four comparisons between with and without endophyte would have been declared as distinct varieties.

Further interpretation of these observations are provided in the section on the frequency of significant differences between with and without endophyte accessions, below.

Table 4. Distribution of significant differences between with/without endophyte between the ryegrass varieties, at Sharnhorst

	Lol A E+	Lol A E-	Lol B E+	Lol B E-	Lol D E+	Lol D E-	Lol C E+
Lol A E+	-						
Lol A E-	ns	-					
Lol B E+	*	*	-				
Lol B E-	**	*	*	-			
Lol D E+	**	**	*	**	-		
Lol D E-	**	**	*	*	**	-	
Lol C E+	**	**	**	**	**	**	-
Lol C E-	**	**	**	**	**	**	ns

ns = no significant differences
** = significant differences p= 0,01
* = significant differences p=0,05 (Not Distinct)

Occurrence of Significant Differences between the definitive stocks of each variety and their submitted E+ and E- Accessions

As reported in the study description section, the EO's included comparisons between the definitive stocks of each variety and the E+ and E- accessions that were provided by the plant breeders. This was an opportunist inclusion given that these definitive samples were already included as part of the control reference collection sown as part of the statutory DUS trials into which the endophyte accessions were incorporated.

In these comparisons there was again considerable agreement between the observations of the three EO's and also across the two species. All three EO's reported significant differences between their definitive stocks and either or both of the E+ and E- accessions of the same variety. For example at Sharnhorst the comparison between Lol D definitive sample and the E+ accession generated three significant differences, two at the $p<0.5$ level (Flag Leaf Length and Inflorescence density), which does not equate to a distinctness, but also one difference at the $p<0.01$ level which does mean that these two samples were distinct from each other (Table 5). This was replicated at Crossnacreevy, where a $p<0.01$ difference was also observed (data not shown), and so a distinctness was again achieved. Similarly, for example, in the comparison between Fes C definitive and E- samples there were four differences observed (Table 6) , three at $p<0.05$ (Character7 – height in spring after vernalisation, Character10 date of inflorescence emergence and Character8 natural height at inflorescence emergence), none of which represent a DUS distinction. However there was a $p<0.001$ difference in Character 'a', natural height in autumn without vernalisation, which made the definitive sample not just highly significantly different from the E- sample, but also represents a DUS distinction.

Table 5. Comparison between the definitive sample of Lol D and an endophyte free sample, at Sharnhorst

Comparison between Lol D definitive and E- following 2 years of testing								
Code No	**Characteristic**	**Mean value**	**Mean diff.**	**T**	**PROB**	**SIG**	**1%**	**5%**
M101	Flag leaf: length	147.52	-22.1	-2.39	3.827	*	29.36	20.64
M102	Flag leaf: width	3.8	0.08	0.42	68.642	NS	0.66	0.46
M105	Flag leaf: length/width ratio	39.6	-6.27	-3.26	0.978	**	6.25	4.35
M201	Plant: length of longest stem, + inflorescence	60.35	2.34	0.66	52.33	NS	11.21	7.88
M202	Inflorescence: length	16.48	-0.58	-0.84	42.457	NS	2.25	1.57
M205	Plant: length of upper internode	18.99	2.78	1.99	7.487	NS	4.42	3.11
M206	Inflorescence: number of spikelets	19.17	0.19	0.51	62.434	NS	1.19	0.83
M207	Inflorescence: density	86.17	-3.65	-2.37	4.197	*	5	3.48
M208	Inflorescence: length of outer glume on basal spikelet	7.35	-0.11	-0.21	84.076	NS	1.69	1.19
M209	Inflorescence: length of basal spikelet excluding awn	11.91	0.45	0.43	67.47	NS	3.3	2.32
M301	Plant: time of inflorescence emergence (after vernalization)	50.73	0.46	0.52	61.602	NS	2.82	1.98
M401	Plant: natural height at inflorescence emergence	46.48	-0.46	-0.23	82.069	NS	6.27	4.40
M402	Plant: growth habit at inflorescence emergence	5.02	-0.11	-0.67	51.988	NS	0.51	0.35
M502	Plant: vegetative growth habit (without vernalization)	6.14	-0.02	-0.15	88.323	NS	0.42	0.30
M506	Plant: width (after vernalization)	36.09	-0.06	-0.07	94.736	NS	3.04	2.14

Table 6. Comparison between the definitive sample of Fes C and an endophyte free sample, at L'Anjouère

Comparison between Fes C definitive and Fes C E- following 2 years of testing														
	2 by 1% Method					COY-D Analysis								
	T Score		Sig Level			Mean Differences						COY-D		
Code No	2013	2014	2013	2014	Sig	2013	2014	Mean	AJ. Mean	Est	T	Prob %	Signif	1%
7	3.59	1.46	1	+	N	5.5o	1.83	3.67	3.67	.	2.20	2.912	*	4.34
11*	0.63	0.64	+	+	N	1.95	2.08	2.01	2.01	.	0.66	50.831	NS	7.93
12	1.81	0.01	+	+	N	0.47	0.01	0.20	0.24	.	0.90	36.748	NS	0.59
14*	3.05	0.31	1	+	N	20.15	2.85	8.87	11.5	.	1.09	27.791	NS	21.26
13	0.56	0.83	+	+	N	4.93	8.36	6.65	6.65	.	0.86	38.98	NS	20.11
B	-1.37	0.64	-	+	N	-28.33	14.21	-7.06	-7.06	.	-0.34	73.638	NS	54.62
10	2.55	1.43	2	+	N	6.66	3.53	5.09	5.00	.	2.01	4.597	*	6.48
8*	-3.69	-0.96	-1	-	N	-4.15	-1.19	-2.67	-2.67	.	-2.19	3.000	*	3.18
a	3.14	2.1	1	5	N	4.33	2.67	3.50	4.25	.	3.46	0.071	***	2.45
All characters were CPVO approved and codes relate to those used by Geves														

For each grass species there were four E- versus definitive comparisons. For ryegrass at Crossnacreevy three of these were distinct comparisons and at Sharnhorst one was a distinct comparison. Furthermore, while none of the four comparisons between E+ versus definitive were distinct separations at Sharnhorst, there was again distinct separation and in the same three varieties as before. For the fescue at L'Anjouère, the E- sample was distinct from the definitive in three of the four varieties and there was also a distinction between the E+ accession and the definitive sample in one of the four varieties. Consequently, in total across all centers and both species the 10 of the possible 16 pair-wise comparisons between the breeders' submitted E+ or E- accession and the EO's definitive samples were reported as distinct comparisons. Therefore, here again if this had been a blind DUS trial these 10 accessions would have been accepted as new distinct varieties.

Result of Microsatellite test

The breeders also conducted a parallel 'Simple Sequence Repeats (SSR) DNA examination of a number of the E+ accessions. A total of 4 SSR markers were used, which were know by experience to be the most informative for describing these endophytes.

The data set was not complete as samples from some accessions were not available from L'Anjouère and there were problems with the DNA purification from some of the plants received by mail from Crossnacreevy. Given this qualification, the evidence derived showed relatively little variation between the endophytes within the endophyte species except that Fes A had different alleles in SSR B11 compared to the other tall fescues (Table 7). There were two varieties that had the same alleles, but as there was only four markers used, this is not definitive proof that the endophytes were identical. It would require further examination using a larger number of markers before the absence of allele difference could be taken as proof that the same endophytes were present. Nonetheless, the fact that 2 endophytes had different alleles for the same marker proves that they were different.

Table 7. Microsattelite Identification of Endophytes in Ryegrass and Fescue varieties

				Microsattelite alleles			
No of plants	**E+ Accession**	**Species**	**Source of Plants**	**B10**	**B11**	**SSR-22**	**SSR-28**
70	Lol B	LP	Eurograss	182	178	148	160
70	Lol D	LP	DLF	182	178	148	160
18	Lol A	LP	Crossnacreevy	182	178	148	0
29	Colosseum	LP	Barenbrug	182	178	148	160
55	Fes D	TF	DLF	166-185	172-198	155-160-174	134
70	Fes C	TF	DLF	166-185	172-198	155-160-174	134
32	Fes B	TF	Barenbrug	0	0	0	0
16	Fes A	TF	Barenbrug	166-185	**150-194**	155-160-174	134
1	Fes A	TF	Barenbrug	166-185	172-198	155-160-174	134
	Control Siegielii endophyte		Control	190	114-125	155-174	180
	Control LP endophyte		Control	182	209	198	182
	Control TF endophyte		Control	164-174-188	150-194	154-158-182	134

Frequency of Significant Differences Between With and Without Endophyte Accessions

The preceding results sections reported a number of distinct separations between with and without endophyte accessions of the same variety, plus a larger number of distinctions reported between the E+/E- accessions and their definitive samples. The question that remains to be answered is whether this evidence indicates that the morphology of ryegrass and fescue plants is so modified by the presence of an endophyte to create a false difference between to accessions of the same variety. Key to this interpretation is an understanding of the likelihood of these distinctions occurring purely by chance or whether they represent a cause and effect.

For ryegrass, the total number of character x variety comparisons was 68 (17 characters x 4 varieties). For fescue, the total number of character x variety comparisons was 36 (9 characters x 4 varieties). It can be seen from Table 8a, that the number of observed differences between E+/E- in ryegrass was less or similar to the expected number purely by chance. The same outcome was found for the comparisons between the fescue E+/E- accessions (Table 8b). Therefore the number of observed distinctions between E+ and E- accessions of the same variety in both ryegrass and fescue were not greater than what can be reasonably expected to occur entirely by chance.

The same examination can also be performed for the comparisons between the definitive samples and the E+ and E- accessions. For ryegrass, the total number of character x variety comparisons was 136 (17 characters x 2 accessions E+/E- 4 varieties). For fescue, the total number of character x variety comparisons was 72 (9 characters x 2 accessions E+/E- x 4 varieties). It can be seen from Table 8c, that the number of observed differences between E+/E- in ryegrass was substantially greater than the expected number purely by chance. Similarly for the comparisons between the fescue E+/E- accessions the observed numbers were much higher than the chance expected numbers (Table 8d). Therefore the number of observed distinctions in both ryegrass and fescue were much greater than what can be reasonably expected to occur entirely by chance.

Table 8. Probability of Expected & Observed

8a) At a sample number of 68 for comparing E+ versus E- in ryegrass

Probability Level	Expected number of differences by chance	Observed Differences at Crossnacreevy	Observed Differences at Sharnhorst
P<0.05	3.4	4	2
P<0.01	0.68	0	1
P<0.001	0.068	0	

8b) At a sample number of 36 for comparing E+ versus E- in fescue

Probability Level	Expected number of differences by chance	Observed Differences at L'Anjouère
P<0.05	1.8	1
P<0.01	0.36	0
P<0.001	0.036	0

8c) At a sample number of 136 for comparing definitive versus E+ and E- in ryegrass

Probability Level	Expected number of differences by chance	Observed Differences at Crossnacreevy	Observed Differences at Sharnhorst
P<0.05	6.8	20	4
P<0.01	1.36	13	1
P<0.001	0.136	6	0

8d) At a sample number of 72 for comparing definitive versus E+ and E- in fescue

Probability Level	Expected number of differences by chance	Observed Differences at L'Anjouère
P<0.05	3.6	23
P<0.01	1.9	10
P<0.001	1.01	4

(Note number of distinctions at p<0.05 includes those at p<0.01 and p<0.001 - similarly for p<0.01)

4. Conclusions

The overall conclusion from the results is that there was no evidence to indicate that the presence of an endophyte had created a false difference when compared to seed from the same seed lot that did not contain endophyte. The SSR analyses did highlight that the diversity of endophytes may not have been particularly wide, which is an important qualification of this overall conclusion. However, the purpose of the study was not to test all possible endophyte species and variants, as this is effectively an unlimited task. Rather the purpose was to test the principle and this was fully achieved. While the study does not preclude the possibility of different endophyte inoculations having a more profound effect, the evidence clearly proves that a change in morphological identity of either a perennial ryegrass or tall fescue varieties is not an inevitable outcome of an endophyte inoculation.

In contrast the large number of differences between the with or without endophyte samples submitted by the breeders compared to the definitive samples held by the EO's, did indicate that there was an identity problem. Although the reported evidence showed that several of the E+/E- samples were distinct from their definitive stock, a role for the endophyte presence was only inferred and not proven. While all the E+/E- samples provided by the breeders came from seed that was originally high in endophyte, it was not the case that both samples gave the same result. Furthermore, the DUS characters that expressed the differences were frequently different in different pair-wise comparisons, which is not consistent with a direct effect of an endophyte infection on the morphological identity of a variety. There is a cause for concern nonetheless as it is unlikely that there were multiple errors in stock maintenance, though there is a possibility that the differences were created in some aspect of the selection of representative plants to create E+ and E- samples and/or during the transport of plantlets to the EO's. The plants that were eventually used for the trsts were the survivors of all of these processes and it is not improbable that some selective drift occurred. It was certainly the case that following an extensive reexamination of the seed lot histories and procedures for creating and maintaining the endophyte that was conducted and reported by the breeders, no clear causal effects could be determined.

Given that this definitive sample comparison was not part of the agreed study with ESA and no clear evidence of an endophyte involvement, these observations should not modify the initial conclusion drawn from the E+ to E- comparative aspect of this study. What has been reported may or may not be a problem for seed certification controls, but this is outside the scope of the current study.

In final conclusion therefore, this study have provided no evidence of a clear link between endophyte presence in either ryegrass or fescue varieties and any change in their identity, sufficient to modify their expression of the CPVO standard set of DUS characters and cause a false awarding of PBR.

5. Next Steps

The final task set for the study team was to offer some scientific backed guidance on CPVO options for future submissions of endophyte inoculated seed. There would appear to be three options open to CPVO as follows:

a) Retain an endophyte free seed requirement

Justification: It can be argued that the DUS test and the subsequent awarding of PBR is based on the plant genotype/phenotype alone. This option will avoid moving away from this base principle to a more complex situation of awarding PBR to symbiotic relationships between eukaryotic and prokaryotic organisms. While the current symbiotic relationship is rather simplified by the effect that morphology and so variety identity is not affected, future examples in other plant species (or maybe even with different endophytes in the same or different grass species) could present more complex problems. Having established the principle that PBR can be awarded to a symbiotic relationship, this might then make decisions regarding future claims more difficult to manage in a different way.

Impediments: To continue to impose this ruling will incur additional work for breeders in providing endophyte free stocks for DUS testing. If indeed any of the observed differences in the current study with matching to the definitive stocks were indeed associated with the removal of the endophyte from E-rich seed stocks, this would create a further difficulty for correctly identifying varieties and protecting them into the future. However, given the absence of any phenotype affects of endophyte presence, at a sufficient level to change variety identity, the breeders fears that certification problems might arise if E- definitive stocks differed from E+ commercial stocks, appear to be nonexistent.

b) Accept endophyte seed applications

Justification: - As there was no evidence in the current study that morphology is affected by the presence of endophytes there are no implications for PBR protection and CPVO can safely allow breeders to submit endophyte rich seed samples. This option also avoids additional work for the breeders in creating endophyte free seed.

Impediments: This option presumes that the experimental observations are universal, ie that they apply equally to all endophyte species and variants and to every grass variety by endophyte/genotype symbiosis. This option will set a precedent whereby CPVO will award PBR to a defined eukaryote/prokaryote symbiosis and also take responsibility for formally registering grass varieties within the EU that contain a microorganism inoculation. The acceptance of this approach by all member states has yet to be ascertained. It may also bring CPVO additional IPR challenges if any endophytes used by breeders are also protected by a patent. Patent IPR could prevent the PBR freedom to breed from registered varieties.

c) Require no information on endophyte presence/absence

Justification: - This option avoids all of the impediments of the first two options and it avoids CPVO formally registering endophyte/plant applications. It avoids setting a precedent of registering a symbiosis and also avoids additional work for breeders. It is fully justified by the absence of clear effects of endophyte presence on variety identity while still leaving CPVO 'silent' and able to react as necessary should a specific grass/endophyte combination prove to modify variety identity in the future.

Impediments: The only specific limitation of this option is that the presence or absence of endophyte in DUS seed submission samples becomes invisible to CPVO. If CPVO wished, however, it could simply require breeders to make a declaration of presence/absence and level of infection, without is having any link to the PBR award. This option also avoids CPVO having to establish levels of infection or setting thresholds that define a 'with endophyte' E+ seed sample and a 'without endophyte' E- seed sample.

Final comments:

It is possibly worth noting that the third option above entirely transfers the 'endophyte issue' to the Value for Cultivation and Use testing for Member State National listing and subsequent EU Common Catalogue listing. This issue was always going to have to be considered by the VCU testers. The third option above in which CPVO takes no action, may make their task less complex as it will leave it entirely in the control of these officials to make the best decisions for their territories.

It is important to note that the recommended options offered in this report refer entirely to the efficacy of the DUS testing system and do not address any wider aspects of endophyte use, including toxicity, patent rights and import phytosanitary regulations across the EU Member States, or even elsewhere.

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ends

Annex 1

Technical protocol of the project trial

Coordinators: CPVO (administrative) and UK (technical) and ESA (breeders)

Contact persons: Anne Weitz (CPVO); Trevor Gilliland (FERA, UK); Bert Scholte (ESA)

Locations: CPVO entrusted examination offices in UK + DE for Lp and FR for Fa

Breeders: ESA - participants will at least include DLF Trifolium (Niels Roulund) and Barenbrug (Stephane Charrier)

Species: Perennial ryegrass; Lolium perenne (Lp), all diploid, amenity

Tall fescue; Festuca arundinacea (Fa), (probably hexaploid)

Varieties: From different origins, per species

1 US + 1 NZ + 2 EU breeding sources, EU registered, 1 maintainer per variety

Endophytes: 2 states of percentage of infection of seeds:

E-free = 0% = E-

E-rich = 100% = E+

Detection absence/presence of E+/E- status by standard method. To be determined for seeds and for the resulting e-rich material in plants, their E+ status confirmed.

Duration: 2 growing cycles (like a regular DUS test)

Nr. of plants: 60 per variety per growing cycle without endophyte infection and

60 per variety per growing cycle with endophyte infection.

(In FR foresee 72 individual plants to be sure to get 60 installed plants for examination)

(note - a different 60 plants must be used per growing cycle)

Propagation: LP plants - Once plants are labelled E+/E-, breeders should split them to provide the cloned plant material needed for the two locations (UK + DE). The plants will need to be tested after splitting in order to confirm their E-rich status before being sent to the testing locations (UK+DE) This is required in order to exclude genetic variation at the 'within' variety level, between the two locations.

Fa plants - no cloning required as only sown at one location

Variables: For each species each year

4 varieties x 2 Endo states (E+/E-) x 60 plants = 480 plants/year

Total for Lp: 2 locations & 2 growing cycles = 480 x2 clones x2 yrs = 1920 plants

Total for Fa: 1 location & 2 growing cycles = 480 x2 yrs = 960 plants

(some additional plants will be required to allow for losses during cloning and transporting, at least 80)

Observations: Trial plots to be included in regular DUS test, to observe and assess the regular DUS characteristics in the relevant CPVO protocol.

Markers: Apply molecular markers for DNA profiling of variety/endophyte combinations (DLF). Markers will be run on the 60 E+ plants for the 4 varieties in one sowing which will give a very good indication of which endophyte(s) are in the investigated varieties. This means that 4*60 = 240 DNA extractions. On each DNA sample will be run 4 different SSR markers on a LICOR gel system giving altogether 960 data points.

In addition the EO's agreed to include the definitive seed samples that they held for each test variety into the study. This was done at no extra cost to the project and at little if any extra cost to the EO's, as these definitive sample were to be sown as part of the reference collection in their PBR trials each year.

Indicative Timetable and Milestones

Timeline	Milestone
2012/10	Start of the project. Preparation of plants by breeders for 1. growing cycle
2013/03	Shipping of plants and planting at EOs
2013/10	Preparation of plants by breeders for 2. growing cycle
2014/06	End of second growing cycle
2014/08	Interim report and meeting (audio or video) for discussions, sending of 1. financial statement from breeders and EOs to CPVO
2014/03	Shipping of plants and planting at EOs
2015/11	Preliminary final report and meeting (audio or video) for discussions,
2016/02	Submission of Final Report (revised milestone agreed by CPVO/ESA given a need for further information gathering following the outcome of the preliminary report and meeting)
TBC	Following acceptance of the final report and closure of the project, exchange of two financial statements from breeders and submission of invoices from EOs to CPVO