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Neotyphodium fungal endophyte in tall fescue (*Schedonorus phoenix*): a comparison of three Northern European wild populations and the cultivar Kentuky-31

Pedro E. Gundel · Marjo Helander · Cecilia Casas · Cyd E. Hamilton · Stanley H. Faeth · Kari Saikkonen

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Abstract Pooideae grasses may be colonized by systemic fungal endophytes. The fitness of endophyte depends entirely on resources and seed transmission from the host plant, while colonized plants may gain increased survival, growth, and reproduction relative to their uncolonized conspecifics. Most research of endophyte-grass interactions have been carried out on few cultivars of tall fescue (*Schedonorus phoenix)* and their symbiont *Neotyphodium coenophialum*. Lack of studies using wild populations of tall fescue across the species natural distribution range, however, limits the understanding of the ecological and evolutionary role of the symbiosis in nature. We performed a common garden experiment in Southern Finland with three wild, tall fescue populations from northern Europe and the forage cultivar Kentucky-31 (KY-31). For each population, we used naturally endophyte-colonized, naturally endophyte-colonized but endophyte removed (decolonized), and naturally uncolonized plants to separate effects due to the host genotype from the endophyte. We evaluated growth variables and survival in four environmental treatments of varying water and nutrients. Supply of water and nutrients increased plant biomass and reproductive effort in all populations. This effect was higher for KY-31 plants which produced on average 55 % more seeds than wild plants, indicating better adaptation to high resource environments. However, the higher incidence of *Claviceps* sp. and the low winter survival indicated KY-31 tall fescue is mal-adapted to Northern European conditions. Naturally colonized plants had greater plant biomass (≈12 %), reproductive effort (≈22 %) and seed mass (≈29 %) than naturally uncolonized and decolonized plants. Nonetheless, endophyte colonization did not affect plant survival, and the effects of endophyte colonization on tiller number, panicle/tiller ratio and *Claviceps* sp. incidence depended on the population origin. In the wild populations, endophyte removal only reduced the number of tillers (≈29 % lower), while the difference between naturally colonized and naturally uncolonized plants was not significant. Our results show that endophyte symbiont increases tall fescue performance in general, but the differences between wild populations and cultivars indicate adaptation to local habitats and agronomic management, respectively. The comparison of naturally endophyte-colonized and decolonized plants suggests certain plant genotype-endophyte combinations found within populations result from local selection pressures.

Keywords Plant-microbial symbiosis · Grass · Symbiosis · Vertically transmitted symbiont · *Claviceps*

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P. E. Gundel (✉) · C. E. Hamilton · K. Saikkonen
MTT Agrifood Research Finland, Plant Production Research,
31600 Jokioinen, Finland
e-mail: gundel@agro.uba.ar

P. E. Gundel
IFEVA - CONICET – Faculty of Agronomy,
Buenos Aires University (UBA),
Buenos Aires, Argentina

M. Helander
Department of Biology, Section of Ecology, University of Turku,
20014 Turku, Finland

C. Casas
Soil Science Division, Faculty of Agronomy,
Buenos Aires University (UBA),
Buenos Aires, Argentina

S. H. Faeth
Department of Biology, University of North Carolina,
Greensboro, NC 27402-6170, USA

Introduction

Grasses of the Pooideae sub-family are the most widely distributed group of terrestrial plants, found from prairies and savannas to high mountains and dunes, and now widely planted in recreational areas, agricultural fields, and pastures (Gibson 2009). The successful performance of these grasses in variable environments may partly be assisted by their microbial symbionts, especially specialized, seed-borne, systemic fungal endophytes (Clavicipitaceae family) (Saikkonen et al. 1998; Clay and Schardl 2002; Tadych et al. 2012). *Neotyphodium/Epichloë* fungal endophytes develop their entire life cycle within the host plant and are dispersed through host seeds (vertical transmission) to subsequent grass generations (Clay and Schardl 2002; Cheplick and Faeth 2009; Schardl 2010; but see Tadych et al. 2012). Anti-herbivore alkaloids and other physiological changes such as higher antioxidant level, associated with endophytes are thought to be responsible for the high frequency of the symbiosis in grass populations and the oft-observed higher fitness of endophyte-colonized plants relative to the uncolonized counterparts (Marks and Clay 1996; Clay and Schardl 2002; Malinowski and Belesky 2006; Saikkonen et al. 2010a; Hamilton et al. 2012; Hamilton and Bauerle 2012). The presumption of endophyte-grass mutualism agrees with evolutionary theory suggesting that vertically transmitted symbionts should be more mutualistic than horizontally-transmitted symbionts since the fitness of both partners, the vertically transmitted fungus and the host grass, are tightly linked (Ewald 1987; Thompson 2005; Saikkonen et al. 2004). However, accumulating evidence indicates fungal effects on host plant fitness are variable and depend on the ecological context (Saikkonen et al. 1998, 2004; Faeth and Sullivan 2003; Cheplick and Faeth 2009; Saikkonen et al. 2010b; Cheplick 2011).

Eurasian perennial grass species tall fescue [*Schedonorus phoenix* (Scop.) Holub. ex. *Lolium arundinaceum*, syn. *Festuca arundinacea*] and perennial ryegrass (*Lolium perenne* L.) are likely to be the most important forage grasses worldwide. They are regularly used for example as turf and for soil stabilization (Cheplick et al. 1989; Gibson and Newman 2001; Hesse et al. 2003; Cheplick 2008, 2011; Hand et al. 2010). Because of their agricultural importance, they have been the subject of extensive breeding programs, and subsequently introduced to cold and temperate regions throughout the world as highly persistent and productive forage. However, these desirable characteristics are offset by the high toxicity to grazing livestock and, in the special case of tall fescue, the invasiveness can threaten the native diversity in successive grassland communities (Clay and Holah 1999; Bouton et al. 2001; Gibson and Newman 2001; Easton 2007; Rudgers and Clay 2007; Gundel et al. 2009; Mattingly et al. 2010). Indeed, the first link between systemic endophytes and toxic syndromes in livestock was discovered in the tall fescue cultivar, Kentucky 31 (Bacon et al. 1977). Because of its economic importance and it widespread distribution, the interaction between tall fescue and their endophytes has stimulated not only agronomic research but also research in ecology and evolutionary biology (Saikkonen et al. 2006). For example, the fungus *Neotyphodium coenophialum*, the common endophyte of tall fescue, promotes tall fescue invasion by inhibiting the establishment of other plant species and thus, affecting community succession and ecosystem functions like nutrient cycling and diversity/productivity relationships (Clay and Holah 1999; Clay et al. 2005; Rudgers and Clay 2007). However, different effects at the population and community level are dependent on abiotic and biotic environments, host genotype and fungal strain (Cheplick et al. 1989; Spyreas et al. 2001; Pecetti et al. 2007; Brosi et al. 2010; Saikkonen et al. 2010b; Vesterlund et al. 2011), like in other endophyte-grass interactions (Cheplick and Faeth 2009). Most of the research on tall fescue-endophyte interactions has used a few cultivars selected for high productivity (Saikkonen et al. 2006, 2010b; Cheplick and Faeth 2009; Cheplick 2011). Because the tall fescue-endophyte interaction is largely the model for the conceptual and general framework for grass-endophyte interactions, it is important to understand how this interaction varies across the natural distribution range of the species.

Dynamics of colonized and uncolonized plants in nature may result from complex processes such as natural selection on one or another phenotype (relative performance), coevolution between partners, variation in the transmission efficiency of endophyte and migrations between populations (Saikkonen et al. 2004, 2010a; Thompson 2005; Gundel et al. 2008). Most studies have focused on the relative performance differences between colonized and uncolonized plants under varying environmental conditions. For example, the fungus may depress tall fescue fitness in resource-poor environments due to metabolic costs (Cheplick et al. 1989), and positive endophyte effects may occur only when environmental conditions are similar to the locality where a certain host genotype and endophyte strain combination has evolved (Hesse et al. 2003; Malinowski and Belesky 2006; Sullivan and Faeth 2008). However, though removal of the endophyte (decolonized) from the host grass has often been used to study those effects ascribed to the symbiosis (See e.g.: Belesky et al. 1987; Cheplick et al. 1989; Marks and Clay 1996; Clay and Holah 1999; Clay et al. 2005), this approach may not provide information on the underlying processes shaping the frequencies and the genetic structure of the colonized and uncolonized plants in populations. To understand endophyte and genetic effects, an experimental approach where naturally uncolonized plants are included as a treatment is required. This is especially important because the frequency of uncolonized tall fescue plants may be variable among natural populations (e.g. Clement et al. 2001; Piano et al. 2005; Gundel et al. 2009; Saari et al. 2010). In natural populations, uncolonized plants may result from genetic incompatibility between partners and/or losses of endophyte from plants and seeds (Saikkonen et al. 2004, 2010a; Gundel et al. 2011). Incompatibility could be important in wind-pollinated grasses since gene flow by pollen exposes the maternally-inherited symbiont to new host genotypes producing genetic mismatches (Saikkonen et al. 2004, 2010a). This process should in the long-term, generate a particular genetic pattern associated with colonized and uncolonized plants in populations. Alternatively, if endophyte transmission failures occur randomly as a result of environmental conditions, then there should not be genetic differences between colonized and uncolonized plants in the population (Gundel et al. 2008, 2011). The interaction outcome of the grass-endophyte symbiosis in nature may vary among populations due to differences in environmental conditions affecting gene flow and ultimately, the coevolution between endophyte and host (Saikkonen et al. 2004, 2010a; Thompson 2005; Morse et al. 2007; Gundel et al. 2010; Hamilton et al. 2010).

Here, we examine how naturally colonized (E+), naturally uncolonized (E−), and manipulatively decolonized (M−) tall fescue plants from wild populations and the KY-31 cultivar from U.S. (the most experimentally studied cultivar; Saikkonen et al. 2006) perform in terms of survival, growth and reproduction under varying resource environments. We hypothesize the positive effect of the endophyte will be evident in the KY-31 cultivar under high level of resources (water and nutrients) but more variable in wild populations. KY-31 is a highly toxic and ecologically aggressive, endophyte-colonized, grass cultivar (Belesky et al. 1987; Clay and Holah 1999; Clay et al. 2005; Rudgers and Clay 2007) that has been selected via breeding programs for high productivity in agronomic systems. Such strong, artificial selection should narrow host genetic variability as well as the single endophyte fungal strain naturally colonizing the host (Saikkonen et al. 2006; Cheplick and Faeth 2009). However, selective breeding for agriculturally desirable traits usually involves trade-offs different from those experienced by plants grown in natural environments (Denison et al. 2003). In addition, the KY-31 cultivar has been grown for more than 70 years in agronomic settings in U.S.A. Therefore we expect KY-31 plants to be mal-adapted to the biotic and abiotic Northern European conditions. Our experimental approach reveals genetic differences (beyond the phenotypic effect due to the endophyte) between naturally colonized and naturally uncolonized plants (Saikkonen et al. 2010a). By varying the resource environment, we tested how environmental factors alter strength and direction of plant-endophyte interaction and how the host plant with and without endophytes responds to different selection pressures.

Materials and methods

Plant material

In August 2003, we collected seeds from wild tall fescue populations at three sites approx. 500 km apart by the Baltic Sea: Åland Island (A: 8 populations), Gotland Island (G: 9 populations) and west coast of Sweden (S: 6 populations). For each population, we harvested seeds from 10 to 50 individual plants. The presence/absence of the *Neotyphodium* endophyte was checked by microscopic examination of three seeds from each individual plant using the staining methods by Saha et al. 1988. All studied tall fescue populations had the seed-borne fungus *Neotyphodium coenophialum* Glenn, Hanlin & Bacon in a varying proportions between 85 % and 100 % of individuals sampled (Saari et al. 2010). For each site (Åland, Gotland, and Sweden), we combined all populations of colonized and uncolonized plant seeds. We also obtained colonized and uncolonized seeds of the Kentuky-31 forage cultivar (KY-31) from University of Kentucky (provided by Dr. T. Phillips). The naturally uncolonized KY-31 seeds were obtained by endophyte removal in the past (T. Phillips, pers. comm.). These colonized and uncolonized KY-31 populations were grown for more than 5 years in different adjacent plots under the same environmental and agronomic management regime.

Manipulation of the endophytic status of plants

To experimentally remove the endophyte, endophyte colonized (E+) seeds from each of the four populations were soaked in warm water (≈ 57 °C) for about 15 min to kill the fungus. This method has proven to be effective in removing the fungus while the seed remains viable (see Saari et al. 2010). In this way, three endophytic colonization treatments were created for each population: naturally endophyte-colonized (E+), manipulatively decolonized (M−) and naturally uncolonized (E−). Eighty seeds from each population origin x endophytic status combination were germinated in Petri dishes (9 mm filter paper, and 5 ml distilled water) under controlled conditions (20 °C and natural photoperiod) in a greenhouse. Seven days after germination, forty seedlings per combination were potted individually in a mixture of sand and peat (50/50, v/v) and kept in the greenhouse until they were transplanted to the experimental field.

Experiment

The field experiment was carried out in Turku Botanical Garden, University of Turku, Finland (60 °26′0″N, 22 °10′ 19″E). When plants from the pots had, on average, 3 tillers, they were transplanted to the field on August 2004. The field site was tilled before starting the experiment. Plants were arranged in a symmetric matrix 0.5 m apart from each other. The experimental design consisted of 10 blocks with 4 plots nested in each, and one individual plant from each population origin x endophytic status combination within the plot. The position of each plant within the plot and the plot within the block was assigned randomly. The experimental area was fenced to exclude large herbivores (e.g. deer, moose, and rabbits), while small herbivores (e.g. voles) could access the area. The space between plants was either hand weeded or sprayed with herbicide (glyphosate Roundup®Bio) twice during the growing season to prevent interspecific competition between weeds and experimental plants.

In 2005, all the plants were double-checked to verify the endophyte colonization status. One leaf sheath per plant was sampled by immunoblot assay to detect specific monoclonal antibodies to *Neotyphodium coenophialum* (Phytoscreen Immunoblot Kit #ENDO7973, Agrinostics, Watkinsville, Georgia, U.S.A.). This in combination with microscopic examination of three seeds per plant that were sampled at the end of the growing season provided a robust determination of the final endophyte colonization status. Water and nutrient treatments were randomly assigned to one of the four plots in each block; treatments were: control (C), water (W), nutrient (N), and combined water and nutrient (W+N). Water treatment plots received 3 L of water per plant three times a week from June through August, and nutrient treatment plots were fertilized with 1 dl of N-P-K (Nurmen Y2, Kemira KnowHow, N-P-K/20-6-6) applied twice during the growing season.

After flowering time, all the panicles were closed in pollination bags (PBS International) to avoid seed loss. The total aboveground biomass from each plant was determined at the end of the growing season (September), by cutting them with a rice sickle at 10 cm above the soil surface. Number of tillers and panicles per plant were counted and seed mass in grams per plant was quantified as was the number of fruiting bodies (stroma) of the pathogenic fungus *Claviceps* sp. This biotropic pathogen causes abortion of flowers (Clay and Schardl 2002) and is used here as a biotic stress factor to study adaptation to local conditions. Plants were dried at ≈70 °C for 48 h to obtain dry matter of plant biomass (g) per plant. During 2006, winter survivorship of plants was recorded.

Statistical data analysis

Total aboveground biomass, the number of tillers, the proportion of reproductive tillers (i.e. panicle/tiller ratio), seed mass and reproductive effort (seed mass/aboveground vegetative biomass) were analysed with mixed effects linear models to account for nested design with blocks, plots within blocks and subplots within plots. Thus, blocks and plots were random factors of the models. The model included population origin, endophyte colonization and environmental treatment as fixed factors. Top–down strategy was applied to get optimal models following Zuur et al. (2009). Likelihood ratio tests for optimal model selection are presented in the “Supplementary material” section. When necessary, the variances were modelled by using specific variance functions (varFunc; Zuur et al. 2009). The ANOVA of the final models (based on REML method) were presented to report the significance of the fixed factors (Supplementary material section). Data transformation was not necessary. Fitted models were checked by plotting standardized residuals against fitted values. Models were run with the lme function (nlme package; Pinheiro and Bates 2009). The incidence of *Claviceps* sp. and plant survival were analyzed in the same fashion by using glmer function (lme4; Bates et al. 2011), which specifies the binomial family (family=binomial (link-“logit”)). Model selection was based on Chi-test nested models and dispersion parameter (phi) was calculated to evaluate the fit (or adequacy) of the model. When corresponded for any model, Tukey’s tests ($P<0.05$) were performed using the glht function in the multcomp package (Hothorn et al. 2008). All the models were conducted in R (R Development Core Team 2011).

Results

Total aboveground biomass per plant depended on the two-way interaction between population origin and environmental treatment ($F_{9,\ 413}=2.25$; $P=0.018$), and on endophyte colonization status ($F_{2,413}=12.53$; $P<0.0001$) (Fig. 1). Plants from the three wild populations responded to the combined treatment of water and nutrients by a 46 % increase in biomass compared to the control. Biomass of KY-31 plants responded to both nutrients alone and water plus nutrients by 30 and 37 % increases, respectively, relative to control (Fig. 1). Endophyte colonization effect on total aboveground biomass was independent of the population origin; endophyte colonization was associated with 7 and 17 % higher biomass per plant relative to that of manipulatively decolonized and naturally uncolonized plants, respectively. However, no difference was observed between manipulatively decolonized and naturally uncolonized plants (Fig. 1).

The number of tillers per plant was affected by the two-way interaction between population origin and the endophyte colonization status ($F_{6,429}=2.40$; $P=0.027$). For the

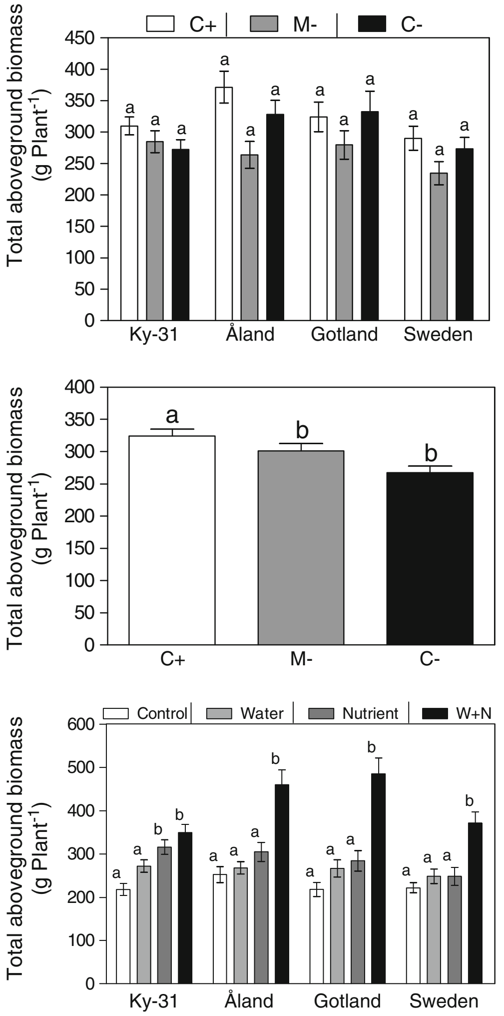

Fig. 1 Total aboveground biomass (g of dry matter) of *Schedonorus phoenix* plants from the different population origins (KY-31, Åland, Gotland, and Sweden) in interaction with endophyte colonization status (*Upper panel*: natural endophyte-colonized: E+, manipulatively decolonized: M−, and natural uncolonized: E−) and environmental treatment (*Lower panel*: Control, Water, Nutrient, and W+T) and the mean effect of endophyte colonization status (*Middle panel*). Values are means ± SEM of $N=40$ (*Upper panel*), $N=30$ (*Middle panel*) and $N=160$ (*Lower panel*). Different letters show significant difference between means within the same population origin (*Upper and lower panels*) and endophyte colonization status (*Middle panel*) ($P<0.05$; Tukey test)

three natural populations, the removal of the endophyte (E+ vs. M−) always meant a reduction (≈28 % less) in the number of tillers per plant but no differences were observed between colonized (E+) and naturally uncolonized (E−) plants. The number of tillers per plant for the KY-31 cultivar was not affected by endophyte colonization status (Fig. 2). The proportion of reproductive tillers (i.e. panicle/tiller ratio) also depended on population origin and the endophyte colonization status ($F_{6,428}=3.71$; $P=0.002$), but was independent of the environmental treatment. This population difference in the proportion of reproductive tillers was only

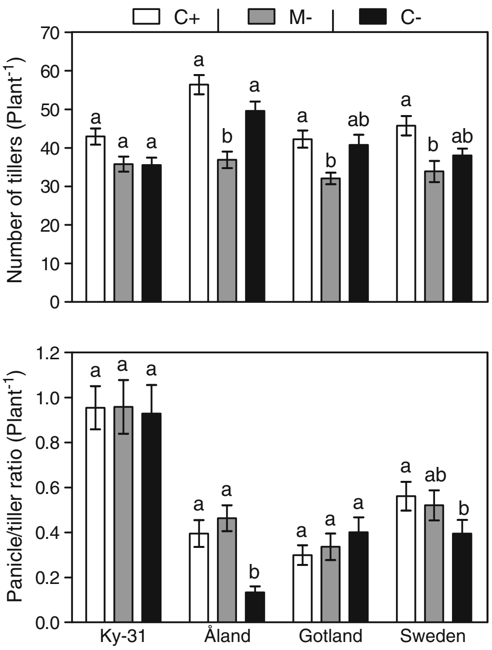

Fig. 2 Number of tillers per plant and the panicle/tiller ratio per plant of *Schedonorus phoenix* as affected by the interaction between population origin (KY-31, Åland, Gotland, and Sweden) and endophyte colonization status (natural endophyte-colonized: E+, manipulatively decolonized: M−, and natural uncolonized: E−). Values are means ± SEM of $N=40$. Different letters show significant difference between means within population origin ($P<0.05$; Tukey test)

significant within Åland and Sweden populations, with naturally uncolonized plants showing a lower panicle/tiller ratio than the naturally colonized and manipulatively decolonized plants (Fig. 2).

Plant reproductive effort was explained by the population origin ($F_{3,353}=17.26$; $P<0.0001$), endophyte colonization ($F_{2,353}=5.41$; $P=0.004$), and environmental treatment ($F_{3,27}=6.02$; $P=0.003$). Gotland plants had a significantly lower reproductive effort compared to the other three populations (≈52 % lower), a higher reproductive effort for colonized plants relative to naturally uncolonized plants (≈22 % higher) and a higher reproductive effort when water and nutrients were added (≈33 % higher than the control) (Fig. 3). Seed mass per plant followed the same pattern as reproductive effort, with significant effects of population origin ($F_{3,364}=14.67$; $P<0.0001$), endophyte colonization ($F_{2,364}=5.88$; $P=0.0031$), and environmental treatment ($F_{3,\ 27}=16.97$; $P<0.0001$) (Fig. 3).

The percentage of plants infected by *Claviceps* sp. stromata depended on the interaction between population origin and endophyte colonization status ($X_6{}^2=22.93$; $P=0.001$) and was independent of the environment ($X_3{}^2=5.24$; $P=0.155$) (Fig. 4). Of a total of 120 plants per population, 18, 9, 6 and 4 showed stromata in KY-31, Åland, Gotland and Sweden populations, respectively. Endophyte colonized plants were the most affected by *Claviceps* sp. in KY-31

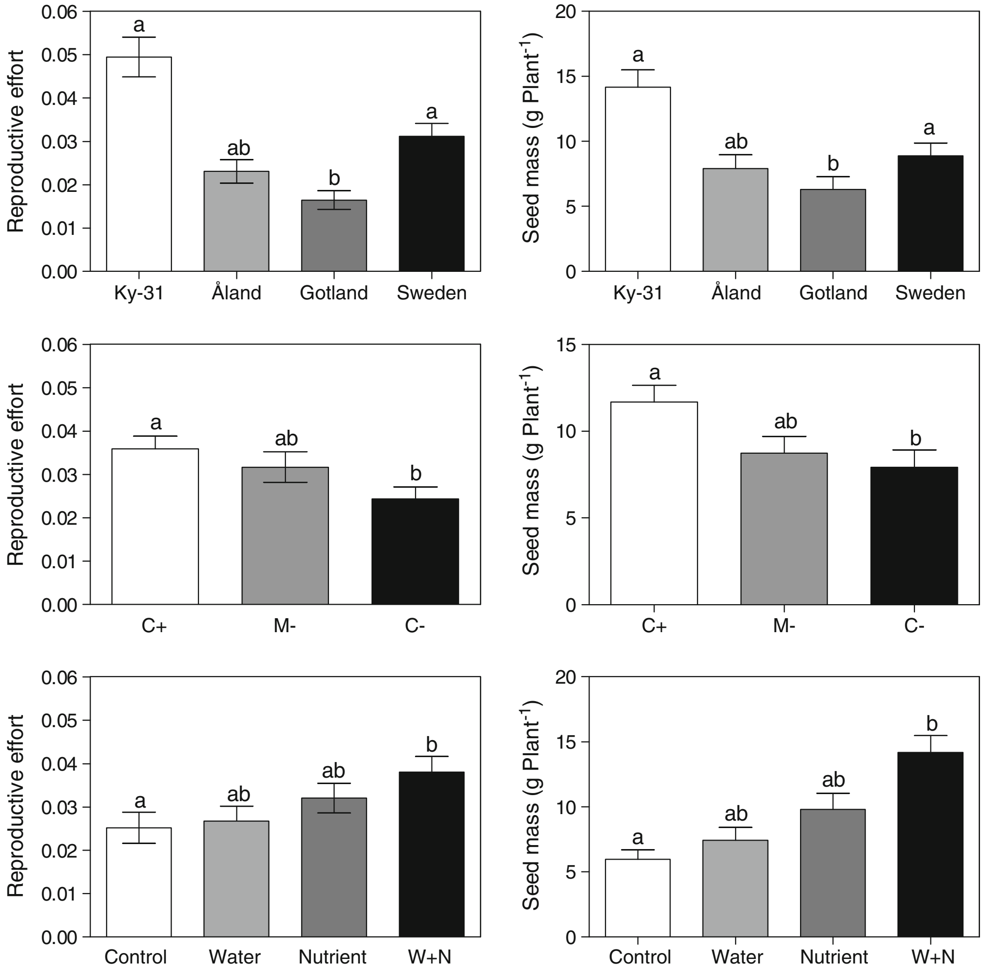

Fig. 3 Reproductive effort (seed mass/aboveground vegetative biomass) and seed mass per plant of *Schedonorus phoenix* from the different population origin (KY-31, Åland, Gotland, and Sweden), endophyte colonization status (natural endophyte-colonized: E+, manipulatively decolonized: M−, and natural uncolonized: E−), and environmental treatment (Control, Water, Nutrient, and W+T). Values are means ± SEM of $N=120$ for population origin and environmental treatment, and $N=160$ for endophyte colonization status. Different letters show significant difference between means within each factor ($P<0.05$; Tukey test)

and Gotland, whereas the reverse was observed in Åland and Sweden where colonized plants showed no pathogenic stromata (Fig. 4).

The percentage of plant survival after winter was affected by the population origin ($X_3^2=20.98$; $P=0.0001$) and the environmental treatment ($X_3^2=14.16$; $P=0.002$), but it was independent of endophyte colonization status ($X_2^2=0.83$; $P=0.661$) (Fig. 5). The KY-31 cultivar and nutrient treated plants had lower survival in comparison to plants from the other populations and those plants that were not treated with nutrients (Fig. 5).

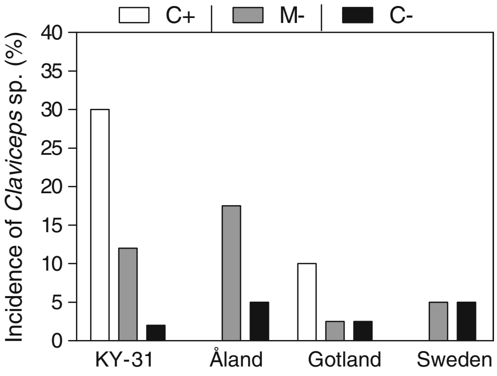

Fig. 4 Incidence of the pathogen *Claviceps* sp. as percentage of plants with at least one stroma, in *Schedonorus phoenix* plants for the different population origin (KY-31, Åland, Gotland, and Sweden), and endophyte colonization status (natural endophyte-colonized: E+, manipulatively decolonized: M−, and natural uncolonized: E−). The number of plants (N) for each combination is 40

Discussion

Outcomes of inter-specific interactions may be affected by the past and current selection pressures, environmental factors operating on the local genetic variability, gene flow between populations, and coevolution between interacting species (Faeth and Sullivan 2003; Saikkonen et al. 2004; Thompson 2005; Sullivan and Faeth 2008; Gundel et al. 2010). Our common garden experiment allowed us to examine the interaction between endophyte colonization and local genetic variation (population origin) in tall fescue populations. Although our results showed this interaction was significant for only some of the observed response variables (i.e. number of tillers per plant and panicle/tiller

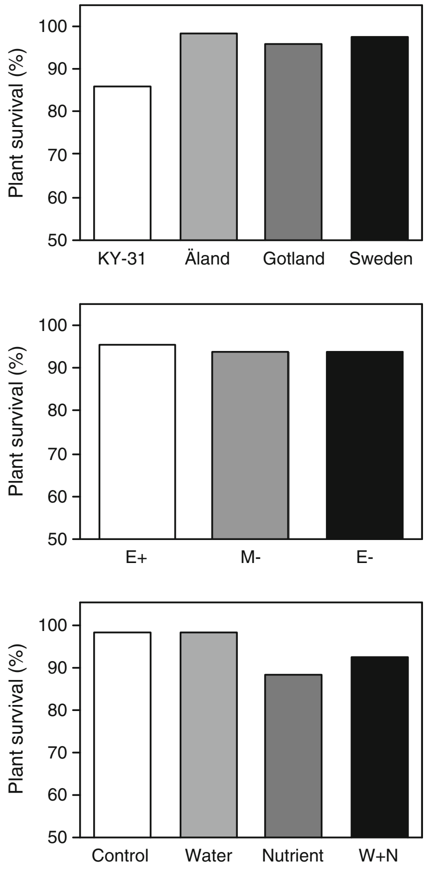

Fig. 5 Plant survival (%) after the winter of *Schedonorus phoenix* plants for the different population origin (KY-31, Åland, Gotland, and Sweden), endophyte colonization status (natural endophyte-colonized: E+, manipulatively decolonized: M−, and natural uncolonized: E−), and environmental treatment (Control, Water, Nutrient, and W+T). Number of plants (*N*) is 120 for population origin and environmental treatment, and *N*=160 for endophyte colonization status

ratio), they reveal geographic variability in the outcome of the symbiotic interaction between tall fescue and the endophyte. Strikingly, although the combined addition of water and nutrients increased plant biomass in all populations, this was not higher for the forage cultivar KY-31 as we hypothesized. However, reproductive effort was higher for the cultivar which produced, on average, 55 % more seeds (g) than wild plants, irrespective of the environmental treatment. The prediction that KY-31 cultivar plants would be mal-adapted to the local Northern European conditions was supported by the higher incidence of the pathogen *Claviceps* sp. and the lower winter survival compared to the local populations. The relatively high infection of KY-31 plants by *Claviceps* sp. may also result, at least in part, from the lack of an evolutionary history of the cultivar to *Claviceps* strains found in Northern Europe.

The lack of a clear difference in total aboveground biomass and number of tillers among population origins and a higher panicle/tiller ratio and reproductive effort in KY-31 compared to wild populations is likely to result from adaptive breeding of KY-31 to agricultural condition in U.S. For example, U.S. and Nordic countries strikingly differ in terms of seasonal changes in temperature and day length which plants can use as environmental cues for adjusting their growth, development and reproduction to local conditions (Heide 1994; Saikkonen et al. 2012). Difference in adaptation to such environmental cues between KY-31 and wild plants may explain the detected pattern. The lack of adaptation to local environment in terms of growth and reproduction could explain the high mortality of KY-31 plants, and may suggest the trade-off between reproduction and survival/lifetime fitness. Another plausible, but not mutually exclusive, hypothesis is that the higher production of new seeds compensates the negative effect in short lifespan of individual plants by high number of offspring enabling the colonization of new habitats. However, despite that endophyte colonization increases host reproductive effort and seed mass, there was no association between the endophyte symbiosis and winter survival of plants. Contrary to the general expectation but in accordance with other results (Faeth and Hamilton 2006; Cheplick and Faeth 2009; Cheplick 2011; Dierking et al. 2012), the endophyte did not have impact on plant survival.

The consistency observed in the phenotypic difference among the endophyte colonization statuses of plants within the three wild populations provides insights into the underlying mechanisms determining the dynamics of colonized and uncolonized plants in nature (Saikkonen et al. 2010a). For most of the evaluated variables (except for panicle/tiller ratio), endophyte-decolonized plants exhibited an intermediate phenotype between the two natural types of tall fescue plants (E+and E−) in all the wild populations. Naturally colonized (E+) plants had, on average, greater plant biomass, higher tiller number, reproductive effort and seed mass compared to naturally (E−) and manipulatively decolonized (M−) plants which is in accordance with the expected alignment in the reproductive success of the symbiotum (i.e. grass-endophyte phenotypic unit; Sullivan and Faeth 2008). Moreover, the effect of colonization depended on population origin for tiller number and panicle/tiller ratio. For the wild populations, we observed a consistent pattern: the number of tillers was negatively affected by the removal of the endophyte (E+: ≈48 tiller/plant vs. M−: ≈34 tiller/plant), but the number of tillers did not differ statistically from naturally uncolonized plants (E−: ≈43 tiller/plant). This suggests that the naturally colonized plants are composite phenotypes of a given host genotype. Thus, the phenotypic difference between manipulatively decolonized (M−) and naturally uncolonized plants (E−) indicates naturally colonized plants (E+) have a different genotype than naturally uncolonized plants (E−). If the random loss of the endophyte in plant and seeds were responsible for maintaining uncolonized (E−) individuals in the populations (Gundel et al. 2011), we would not expect genetic differences between naturally colonized and uncolonized plants. The different plant genotypic pattern between naturally colonized and uncolonized plants within populations may results from gene complexes governing the partners' compatibility or from local coevolution between host plants and endophyte symbionts (Saikkonen et al. 2004, 2010a; Thompson 2005; Gundel et al. 2010; Hamilton et al. 2010).

The native range of tall fescue covers Europe, East of Asia and Northern Africa, and includes a wide variety of natural environments like dry Mediterranean grasslands, damp meadows, river banks, and seashores (Tutin et al. 1980; Gibson and Newman 2001; Inda et al. 2008; Hand et al. 2010). Phylogenetic and geographic studies reveal that, at a large scale, three different major morphotypes of tall fescue are identified (Mediterranean, Continental and Rhizomatous) which differ in their growth form and seasonality in response to their native range (Inda et al. 2008; Hand et al. 2010). The most important forage cultivars worldwide have arisen from the Mediterranean (Southern Europe and North Africa) and Continental (Northern Europe) morphotypes, and the cultivar KY-31 in particular, is a summer-active Continental morphotype (Hand et al. 2010). Therefore, the differences we found may be related to genetic diversity within the Continental morphotype. Indeed, the wild populations in our study may be distant ancestors of the KY-31 cultivar. Further, it seems to be clear that, at the same large scale, there are differences in the fungal endophytes associated with each morphotype (Hand et al. 2010). Nonetheless, a survey of tall fescue wild populations around the Mediterranean has shown a relatively high endophyte genetic diversity in natural populations (Piano et al. 2005), which contrasts with the low diversity associated with cultivars (Saikkonen et al. 2006; Morse et al. 2007). Therefore, considering the higher gene flow rate of grasses (by means of seed and pollen) relative to the lower gene flow of vertically transmitted fungal endophytes (largely restricted to the seeds) (Saikkonen et al. 2004; Gundel et al. 2010), it is conceivable that at least part of our results are due to genetic differences in the endophyte. This higher genetic diversity in the wild populations provides the raw material for partners of coevolution and geographic variability in the symbiotic outcome of grasses and systemic endophytes (Saikkonen et al. 2004; Piano et al. 2005; Thompson 2005; Gundel et al. 2010).

Unlike the evolutionary dynamism of the symbiosis in wild environments, accumulating evidence indicates that, for the agronomic grasses tall fescue and perennial ryegrass, host plant genotype often explains a large fraction of the response of host plants to the environmental conditions (Pecetti et al. 2007; Cheplick 2008; Dierking et al. 2012). For example, Mediterranean populations performed better under Mediterranean conditions, and Continentals performed better under continental conditions in Italy, with endophyte only marginally improving the plant fitness under Mediterranean conditions (Pecetti et al. 2007). Similarly, endophyte removal from other Mediterranean and Continental cultivars had little influence on the physiological traits and plant survival to freezing temperatures (Dierking et al. 2012). However, the fungal strain or endophyte haplotype has been also found to have a stronger effect than simply whether a plant is colonized or not, at least in wild populations (Morse et al. 2007; Hamilton et al. 2010; Sullivan and Faeth 2008). Therefore, the performance of agricultural forage grasses seem to rely mostly on host adaptation possibly to only one or just few, fungal strain, while the success of the grass-endophyte symbiosis in the wild is a complex process dependent on adaptation of the partners to each other and to the variable environmental conditions (Saikkonen et al. 2004; Morse et al. 2007; Gundel et al. 2010; Hamilton et al. 2010). The hypothesis that host plants and the fungus work in concert as a phenotypic unit will remain to be tested in future studies using manipulative experiments incorporating molecular tools. Such studies may reveal the association between vertically transmitted endophytes and a unique host genotype or maternal lineages within wild populations.

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