

Grass Pollen Pollution from Biofuels Farming

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Based on its low costs and high harvests, red fescue (*Festuca rubra* ssp. *rubra* L.) is a promising plant for biofuel production in the subtropics and temperate climate. Once the acreage increases rapidly, its pollen pollution was studied in detail. Robust analysis on the shape, size, and chemical composition, especially the presence of allergens was conducted for 2 successive years in relation to 3 levels of N- and PK-fertilization. Multiparametric statistical analysis showed that there was no significant effect on the pollen grain size nor on the profile and content of the phenolic compounds. On the other hand, intense fertilization naturally increased the number of panicles, and consequently the overall pollen production. In addition, higher levels of fertilization significantly ($P_{0.016}$, resp. $P_{0.011}$) influences the content of tryptophan, which is involved in the occurrence and course of the allergic reactions.

Introduction

FARMING OF GRASSES (*Poaceae*) is becoming more popular since it requires relatively low costs and the biomass obtained may be profitably utilized in energy production.^{1–4} However, during the flowering season, the pollen is released from the anther as a dehydrated microspore containing the male gametophyte. It is spread by wind as a pollen cloud, which dilutes rapidly with increasing distance.⁵ Gramineae pollen is the most frequent agent of pollinosis (hay fever, popularly), but it can also cause asthma and other health problems. Numerous cross-reacting pollen allergens have also been proven in persons sensitive to certain foods (eg, peppers, tomatoes, kiwi, celery, onion, pea, and peanuts), often resulting in oral allergic syndrome. All of them trigger a response by the immune system, even with a concentration of 5 to 50 pollen grains per m³. This allergy is seasonal, as well as regional, depending on the pollen quantity in the air. Although *Poaceae* is the fourth most abundant family among flowering plants with respect to the number of species, only 20 species of that enormous number (more than 10,000 species) show sensitization effects, among them red fescue.^{6,7} Allergens in red fescue are often referred to as Fes p 1, 4, 5.

Although red fescue copes with a low level of fertilization, seed growth requires proper species management, particularly, proper nitrogen fertilization already in late summer. The aim of this study was to examine whether different levels of nitrogen nutrition are related to the content of specific compounds in the pollen grain within the context of allergic effects and how the farmers would manage the knowledge obtained.

Materials and Methods

Experiment site

The experiment was carried out in the field of the plant breeding station of TAGRO, Ltd. at an altitude of 420 m, with a mean annual temperature of 7.9°C, and mean annual precipitation of 590 mm. The soil type is Cambisol acid, with a soil texture of loamy sand on gneiss substratum, pH_{CaCl2} 5.15, CEC mean (180 mmol⁺/kg), C_{ox} 14.6 g/kg, N_{NH4} 4.9 mg/kg, N_{NO3} 21.3 mg/kg, and available nutrients (Mehlich 3) P 154 mg/kg, K 407 mg/kg, Mg 125 mg/kg, and Ca 1,385 mg/kg.

The field experiment was established in the spring of 2009 as a small plot experiment with red fescue (*Festuca rubra* ssp. *rubra* L.), cultivar TAGERA, with a plot area of 10 m², and variants in 5 replications. The method of establishing and managing the experiment was the standard according to the official methodology of the Central Institute for Supervising and Testing in Agriculture (UKZUZ) for State Variety Tests.⁸ In the course of testing, no pesticides were used. The trial was evaluated in the first 2 harvest years.

Primary fertilization (presowing). In Treatment A, there was no fertilization, while in Treatments B and C, the first fertilization took place before sowing; in further crop years, fertilizer rates were applied at the same level.

Fertilization variants. A: (check) without any fertilization;

B: (a lower level of nutrition) with a half nitrogen rate: application of 70 kg N ha⁻¹ (in the spring, 30 kg N ha⁻¹ at a growth height no more than 100 mm; 40 kg N ha⁻¹ after seed mowing, that is, in ½ VIII–½ IX at a new growth height of 40–100 mm) + [40 kg P ha⁻¹; 60 kg K ha⁻¹] per year.

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C: (a higher level of nutrition) with a full nitrogen rate, that is, double the rate of Treatment B + [40 kg P ha⁻¹; 60 kg K ha⁻¹] per year.

Pollen collection in both the first and second crop year (10/6/2010; 8/6/2011) was carried out at the time at which the number of flowering florets in the vegetation reached its peak (3rd day of flowering), from 5:30 to 7:00 p.m., at a temperature of 30°C, with no wind, using a sterilized vacuum cleaner (suction of 8 kPa). Pollen at the correct moisture content was captured in special paper bags and transported to the processing point within 2 h.

The treatment of the collected pollen is carried out with standardized protected technology in a laboratory environment using sterilized machines.⁹ This way, the storage of treated pollen can be guaranteed for ≤2 years (in hermetically closed containers, without light, and at a given temperature). Collected pollen from individual treatments and replications is finally dried separately in heaters (72 h at 32°C). Treated pollen from each plot with 6% moisture was divided into 2 halves, whereas the first half was used for allergology analyses and the second for chemical analyses.

The pollen grain size was measured using a scanning microscope. Five grains from each plot in both crop years were measured.

Extraction of phenolic compounds from processed pollen

The exact weight of pollen was in the range of 0.2 g. The pollen was first extracted by hexane for the removal of nonpolar compounds. The hexane fraction was discarded; the sediment was dried and extracted for 30 min by intensive shaking at laboratory temperature with 2 mL of 70% methanol. After centrifugation (3,500 rev/min, 10 min), the supernatant was removed and the sediment was extracted once again for 15 min by 70% methanol. After a second centrifugation, both supernatants were combined and the exact volume was noted. The combined extract was stored at -18°C until measurement.

HPLC analyses

The separations were performed on a Hewlett Packard HPLC system, Model 1050, with an Agilent (model G1315B) diode-array detector, using a Luna Phenomenex C18(2) column (150×2 mm, 3 μm). The injected volume was 5 μL. Mobile Phase: water+acetonitrile+*o*-phosphoric acid. Mobile Phase A: 5% acetonitrile+0.1% *o*-phosphoric acid; Mobile Phase B: 80% acetonitrile+0.1% *o*-phosphoric acid. The following gradient was used: from 0% to 45% of Mobile Phase B in 55 min and from 45% to 100% in 10 min, with a flow rate of 0.25 mL/min, at 25°C. Phenolic compounds were detected at 220 nm, and the concentration of selected compounds was calculated from the routine calibration curve.

LC-MS analyses

The measurements were performed on an LC-MS LCQ Accela Fleet (Thermo Fisher Scientific) equipped with an atmospheric pressure chemical ionization source and a photodiode array detector using a Hypersil Gold column (2.1×50 mm, 1.9 μm) and a mixture of water-acetonitrile-formic acid as a mobile phase. Mobile Phase A: 5% acetonitrile+0.1% formic acid; Mobile Phase B: 80% acetonitrile+0.1% formic

acid. The gradient was increased from 0% of B to 45% of B in 10 min, and the flow rate was 0.400 mL/min.

Protein profile and total allergenic activity

Allergen extraction. Ten percent ET IIF extracts were prepared from every pollen sample (bicarbonate buffer with phenol addition, pH 7.8–8.4) after 24 h at 2°C–8°C, and then they were filtrated through the prefilter and membrane filters of 1.2–0.22 μm. A protein profile was established by means of electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS)—SDS-PAGE, and the presence of general allergens was established qualitatively according to immunoblotting, and the total biological (allergenic) activity by means of the *in vitro* method of ELISA inhibition assay.

ELISA inhibition assay. The principle of this method is linking a specific immunoglobulin E (IgE) preincubated with the examined allergen in the concentration series (binary dilution) to an allergen tightly linked to the base. The concentration of specific IgE (unsaturated by the examined allergen and therefore linked to the imbedded allergen) is determined by means of an anti-IgE antibody tagged by an enzyme (horseradish peroxidase) and the subsequent reaction of this enzyme with a chromogenic substrate. The color intensity is then directly proportional to the examined allergen concentration (allergen epitopes, resp.). The assessment is carried out by means of an ELISA reader.

SDS-PAGE

SDS linked to proteins unifies their charge so that the final mobility of proteins is only dependent on the size of their molecule. The evaluation is carried out by the comparison of electropherograms of the sample extract and the standard (standards of molecular weights, resp.). Samples are correct, if they show the same protein profile in the electropherogram, that is, they do not differ in the number and location of zones and similar series of allergens can be assumed. Important values of molecular weight are ca 17, 30, and 70 kDa (where the majority of allergens are located). The evaluation is carried out in an electrophoresis device for vertical electrophoresis in a polyacrylamide gel.

Statistical analysis

For statistical computation, we used R, free statistical software (The R project for Statistical Computing). Because the eight pollen compounds could in fact be correlated random variables, we decided to use the multiparametric version of the analysis of variance (ANOVA) test—multivariate analysis of variance (MANOVA). More precisely, we tested the hypothesis that nitrogen fertilization has no effect on pollen compounds under the following 4 MANOVA test statistics: Wilks's, Pillai's, Roy's, and Hotelling-Lawley's.

Results and Discussion

The analyses showed that the red fescue pollen grains are yellow, smooth, and round in shape, measuring 36.4 μm (with a range of values of 33.8–37.1) on average. The differences among fertilization treatments were not significant ($P_{0.05}$), nor were differences among harvest years ($P_{0.05}$). Eight compounds, which were dominant on the chromatogram, were qualitatively evaluated by HPLC (see Table 1),

TABLE 1. CHROMATOGRAPHIC AND MASS SPECTRAL DATA OF INVESTIGATED COMPOUNDS

Number of compound	Retention time on chromatogram (min)	Identification under DAD Spectra	Ions in full scan (+APCI)
1	12.0	Tryptophan	205 ⁺
2	14.8		402 ⁺ , 360 ⁺ (M-42), 342 ⁺ (M-42-H ₂ O)
3	18.5		374 ⁺ , 195 ⁺
4	23.8	Quercetin diglucoside	627 ⁺ , 465 ⁺ (M-162), 303 ⁺ (M-162-162)
5	25.1	Rhamnetin (isorhamnetin) diglucoside	641 ⁺ , 479 ⁺ (M-162), 317 ⁺ (M-162-162)
6	26.3	Quercetin diglucoside	611 ⁺ , 465 ⁺ (M-146), 303 ⁺ (M-146-162)
7	28.8	Kaempferol diglucoside	595 ⁺ , 449 ⁺ (M-146), 287 ⁺ (M-146-162)
8	45.6	Caffeic acid derivative	796 ⁺ , 761 ⁺ , 515 ⁺

DAD, diode-array detector.

while their mass spectral data (molecular and fragment ions) was also given. By combining the UV spectra of the standard compound and the mass spectral data, we were able to confirm Peak No.1 as tryptophan, and further to assign Peaks Nos. 4 through 8 to the group of flavonols and/or caffeic acid derivatives. The most significant fragment in the mass spectra (APCI-positive mode) of compound Nos. 4 and 5 is the fragment with an m/z value of 162, which belongs to anhydroglucose and is typical of the fragmentation of glucosides. According to the fragmentation of Peak No. 4 (M-162-162), the compound is quercetin diglucoside. Similar fragmentation was also seen in Peak No. 5 (M-162-162), with the difference in the molecular peak being 14 amu higher in comparison to Peak No. 4. This means that Peak No. 5 could be rhamnetin or isorhamnetin diglucoside. The most significant fragment in the mass spectra of the next 2 compounds (Nos. 6 and 7) is the fragment with an m/z value of 146, which belongs to deoxyglucose, or most probably to rhamnose. Therefore, Peak No. 6 is quercetin diglucoside, in which one sugar unit is deoxyglucose, or most probably rhamnose. Although Peak No. 7 has a similar fragmentation pattern (M-146-162) to the previous quercetin diglucoside (Peak No. 6), the molecular peak M^+ has an m/z value of 286, which is 16 amu less than the value for quercetin (m/z 302). This means that compound No. 7 only has 4 hydroxy groups compared to quercetin. Therefore, compound No. 7 is most likely kaempferol diglucoside; on the basis of the UV spectra,

we excluded luteolin diglucoside as a possible next candidate from the list of compounds. The phenolic compound content in the extract (Fig. 1) was not significantly influenced by the nutrition level ($P_{0.05}$) nor were the differences among years (2010, 2011) significant ($P_{0.05}$). Whereas the contents of these compounds are remarkably stable in pollen, in a vegetative mass of red fescue, they are markedly variable according to the growth phase, nutrition level, etc. For example Štěrbová et al.¹⁰ have identified in the plant the following phenolic acids: protocathechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, *p*-coumaric, ferulic, and rosmarinic acid; further, *p*-hydroxybenzaldehyde and vanillin. In the plant of red fescue, Fossen et al.¹¹ also described cyanidins, peonidins, and delphinidins. Generally, the contents of compounds in the generative part of the plant vary by the effect of exo- and endogenous factors significantly less than in vegetative parts, particularly at the time of the full ripeness of the grains, because they are biologically a final product of the plant and their biological development is finished.¹² Actually, pollen also bears signs of a finished cycle within the framework of its biological mission. Further, the effect of various fertilization rates on the biological (allergenic) activity of pollen extracts in comparison with the intradepartmental (study) standard of SEVAPHARMA a.s. has been assessed. The results proved the same protein profile, including a significant presence of majority allergens in the area of molecular weight (mw) 17, 30, and 70 KDa

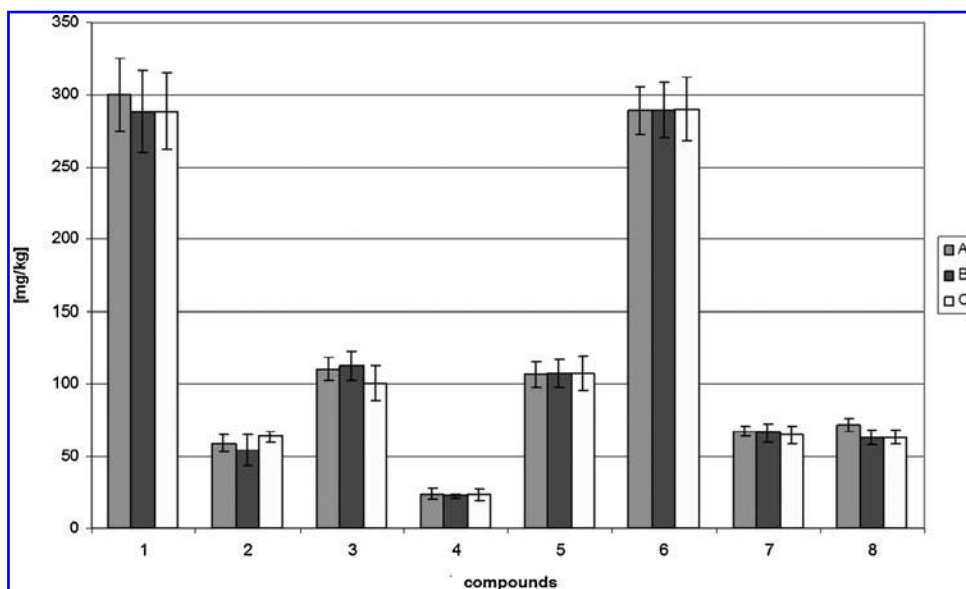


FIG. 1. Tryptophan and phenolic compound content depending on the fertilization level 2010 and 2011 Means. A, unfertilized; B, lower fertilization level; C, higher fertilization level.

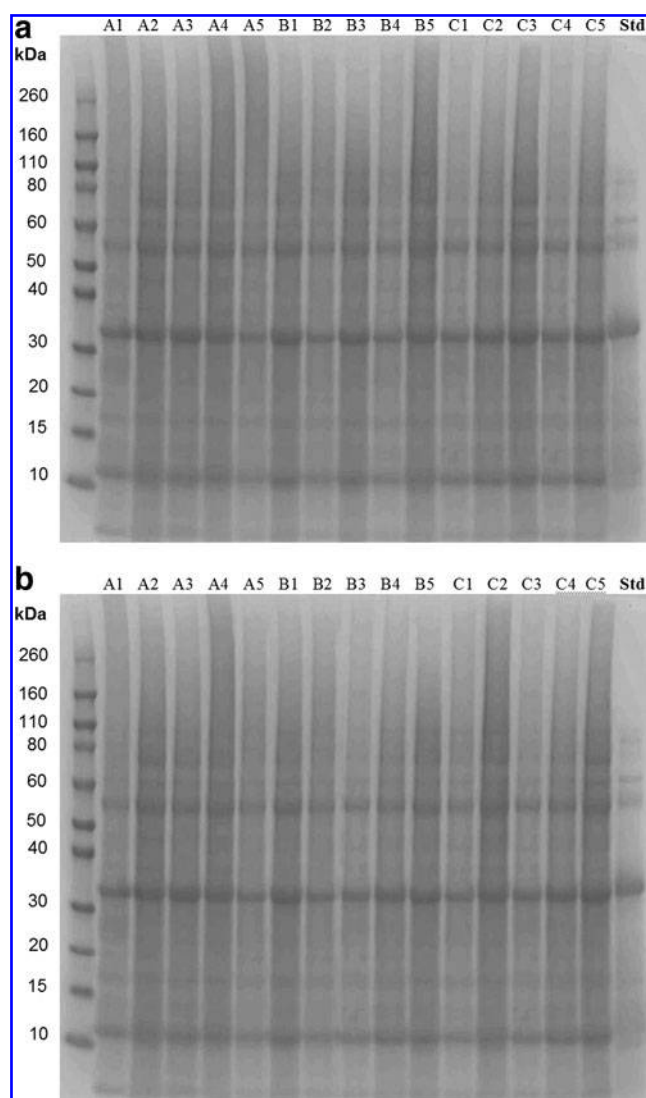


FIG. 2. SDS-PAGE-protein profile from Pollen of Red Fescue cv. TAGERA (v kDa). **(a)** 2010 Pollen Harvest. **(b)** 2011 Pollen Harvest. Std, standard; A1, 2, 3, 4, 5, fertilization level A—5 replications; B1, 2, 3, 4, 5, fertilization level B—5 replications; C1, 2, 3, 4, 5, fertilization level C—5 replications.

(Fig. 2). The differences among fertilization treatments were not significant ($P_{0.05}$), nor were differences among crop years ($P_{0.05}$).

At the time of flowering, the crop of red fescue contributes to the allergic risk increase in a given region. Even though the pollen allergenicity itself was not influenced by the various fertilization levels of growth, higher fertilizer rates generally led to a panicle number increase, and thus higher pollen production.¹³ One grass panicle, for example, produces up to 5 million of these grains. If the crop is placed in an area of markedly polluted air and consequently soil, there occurs an additional factor of a higher amount of CO_2 , which affects the total pollen production and increases the allergenicity potential in pollen grains, not only for Ambrosia, but probably for grasses too.¹⁴ By means of partial MANOVA test statistics, we found in the set of analyzed compounds that along with the fertilizer rate increases the content of tryptophan in pollen grains. It is most significantly

shown in Roy's statistics ($P_{0.016}$, $P_{0.011}$, resp.), but not its biological activity ($P_{0.05}$). Since the human population ingests this essential amino acid solely with food, the consumption of products containing pollen (food and beverages contaminated by pollen) with increased tryptophan content could produce an increase in the tryptophan concentration in the blood, and thus also a lower degree of effectiveness of allergic rhinitis treatment in patients treated by means of specific immunotherapy.¹⁵ Tryptophan metabolism is involved in the occurrence and course of the allergic reaction, even if the mechanism increasing blood concentration is not yet precisely known. The reactions of farmers informed about these findings are as follows: the majority of farmers behind the town Tábor (Czech Republic) have no idea about the consequences of fertilizing and pollen production. On the other hand, they are aware of bad public relations when farming in urban areas and they would agree on lowering the levels of fertilization and lower profits than any unpleasant problems with citizens from the neighborhood.

Conclusions

The level of fertilization did not affect the shape or size of the red fescue pollen. The phenolic compound content was not significantly influenced by the nutrition level. Eight major compounds were qualitatively and qualitatively evaluated in the pollen. Whereas the amount of these compounds varied in the vegetative mass according to the nutrition level, only the amount of tryptophan increased with higher nutrition levels in the pollen. Knowing that the increased concentration of tryptophan in blood reduces the effectiveness of allergic treatment, and that the overall role of tryptophan in human organism is not sufficiently understood, we conclude with a precaution that grasses farmed nearby dwellings over the flowering season should not be fertilized.

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Author Disclosure Statement

No competing financial interests exist.

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