Phleum pratense-pollen adaptive variations and pollen microbiome investigation under different climatic regions and prospects of allergenicity

Muhammad Humayun
Saadia Naseem
Richard E Goodman
Zahid Ali (zahidali@comsats.edu.pk)
COMSATS University Islamabad https://orcid.org/0000-0003-3935-6816

Research Article

Keywords: Climate change, P. pratense, Biolog, Brevibacterium epidermidis

Posted Date: October 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3358447/v1

Abstract

Phleum pratense is an allergenic grass that pollinates in spring. Databases Allergenonline.org and Allergen.org record ten *P. pratense* allergens and their isoforms. Phl P 1, Phlp 5 and Phl p 11 are major *P. pratense*-pollen allergens with demonstrated basophil activity and skin test reactivity. Little is known about *P. pratense* pollen adaptive variations in different climatic regions and pollen associated microbial diversity. In this study, we collected *P. pratense*-pollens in the spring season 2022, from three climatic regions (R1, R2 and R3) in Pakistan having difference in mean annual air temperature, mean annual precipitation and elevation. The morphology of pollens was observed by light microscopy, scanning electron microscopy (SEM), biochemical fingerprint analysis and composition of pollens were investigated by fourier-transform infrared spectroscopy (FTIR). The pollen-associated bacterium was identified through Biolog GEN III microplate system. The pollen water-soluble proteins were isolated and stabilized in phosphate buffer saline (PBS) and tested for allergenicity response through dot blots and western blots analysis. Morphological study found difference in pollen biochemical composition. Biolog identified *Brevibacterium epidermidis* from *P. pratense* pollens. Protein extracts quantification and sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) gel found decreased protein expression in R1 region pollens in comparison to R2 and R3 regions pollens. Allergenicity studies found differential expression of beta-expansin and profilin (allergens) in pollens obtained from three regions. Beta-expansin and profilin were suppressed in R1 pollens, and expressed in R2 and R3 pollens. This is the first study to identify *B. epidermidis* growth on *P. pratense* pollen. A variable allergen expression in *P. pratense* pollens has also been observed in different regions. An increase in mean annual temperature and decrease in mean annual precipitation affected pollen biochemical composition, and inhibited beta-expansin and profilin expression involved in pollen growth and development. Therefore, the findings of the research are unique, which enhances basic knowledge and understanding of *P. pratense*-pollen associated microbiota and climate change impacts on the pollen allergen expression.

Introduction

Phleum pratense (Timothy grass), a member of *Poaceae* family, grows in the sub-tropic regions of the Northern Hemisphere (Xu et al. 2021). Its pollens cause allergies in susceptible populations in many countries (Sekerkova et al. 2012; Mendy and Zeldin 2020; Beggs 2021; Xu et al. 2021) and accounts for 15% and 20% grass pollen sensitization in the United States of America and Europe (García-Mozo 2017). The grass single pollen contains 0.040 ng allergen that activate human immune system (Jung et al. 2021). Water-soluble proteins of the pollen encounter the mucosal layer of the nasal cavity during inhalation, and the induce immune system, resulting in sneezing and release of watery fluids from the nasal cavity. So far, 10 allergens (Phl p 1, Phl p 2, Phl p 3, Phl p 4, Phl p 5, Phl p 6, Phl p 7, Phl p 11, Phl p 12, and Phl p 13), and their isoforms reported from the grass pollen (Allergen.org), (García-Mozo 2017). Out of these 10 various pollen allergens, Phl p 1, Phl p 2 and Phl p 5, cause 50–95% *P. pratense* pollen allergies (Hoffmann et al. 2022). Individual pollen allergens of the grass are very well characterised and specific bacterial communities from the pollen have been reported (Obersteiner et al. 2016). The birch tree

and *P. pratense*-microbial diversity correlation to allergenicity factors have been identified (Bet v 1/ Phl P 5 pollen associated lipid mediators). Inhalation of bacterial endotoxins (lipopolysaccharide) has been reported in lung inflammation (Cardinale et al. 2019). Similar to lipopolysaccharide gram-positive bacteria supernatants may induce allergic immune responses in human beings. Dysbiosis of the ocular surface microbiome plays an important role in ocular pathogenesis (Cavuoto and Zhu 2022). Epithelial layer dysfunction role has been indicated in rhinitis, atopic dermatitis, food allergies and asthma (Singh et al. 2022) coeliac disease, eosinophilic esophagitis and inflammatory bowel disease (Akdis 2021). *P. pratense* pollens growing in different climatic conditions may contain microbiota that may be involved in pollen allergy.

P. pratense grows in different climatic regions of the globe and the allergen expression of *P. pratense* under different climatic conditions is important to investigate climate change effect on the grass pollen-allergen expression. Climate change is one of the biggest global challenges (Beggs 2021), and studies have indicated that it affects pollen chemical composition (Zimmermann et al. 2017), pollination season and pollen allergies (Subiza et al. 2019)(Sapkota et al. 2019, 2020; Beggs 2021). Water stress in cereals causes pollen infertility, and an increase in temperature (~ 5°C) above the optimum growth conditions of a plant inhibits mRNAs and proteins synthesis (Shyam S. Mohapatra 1996). Temperature increase causes immature bursting of the tapetum layer of the pollen due to oxidative stress that makes pollen infertile. Oxidative stress in ryegrass a member of *Poaceae* due to variant pollution levels affects pollen allergenicity (Lucas et al. 2019). Ryegrass responds differently to drought and elevated air temperature. *P. pratense* produced less pollen allergen content in response to drought and elevated air temperature (Jung et al. 2021). Pollen biochemical composition (proteins, lipids and carbohydrates) adaptation varies in response to different environmental conditions, especially temperature (Zimmermann et al. 2017). Climate change impact on aeroallergen growth and development, concentration and distribution, and associated allergies depict increase in asthma and allergy patients hospitalizations (Beggs 2021). The climate change impact on *P. pratense*-pollen allergen expression and their biochemical composition sensitization is not studied so far. The grass grows in different climatic regions of Pakistan, which is reckoned among the top most affected countries due to climate change. This study explores adaptive variations in *P. pratense* pollen and associated allergies, and *P. pratense* pollen-associated microbiome in three different climatic regions of Pakistan. We profile that difference in mean annual temperature, mean annual precipitation, and elevation from sea level affect pollen morphological features and allergen expression.

Materials and methods

Pollen Sampling

P. pratense pollens were sampled in March 2022 from three distinct geographic regions of Pakistan. Region 1 (district Peshawar), region 2 (district Islamabad) and region 3 (district Kotli) located at 331 m, 507 m and 609 m elevation from sea level respectively. Region 1 has hot weather, region 2 and 3 have moderate weather conditions. The mean annual temperature during 2010–2020 for region 1, region 2 and

region 3 was 23.06°C, 22.07°C and 21.39°C, and mean annual precipitation during the period was 42.31 mm, 102.445 mm, and 110.34 mm respectively (https://www.pmd.gov.pk/en/). For each region, mature pollens were collected from 3–5 nearby-located *P. pratense* plants on a sunny day by clipping the inflorescence of the plant in sterile 50 ml falcon tubes (Humayun et al. 2023). Samples were air dried at room temperature for one week and then tipped on a 500-µm pore size mesh. Isolated pollens from the inflorescence were passed on a 100-µm pore size mesh to remove non-pollen floral parts and optimize pure pollen concentration. Pure pollen samples were stored at room temperature in 1.5 ml sterile tubes.

Microscopy

Pollen grains (10 mg) were soaked in iso-propyl-alcohol (200 µl) for ten minutes. Then 10 µl of the mixture was spread on a microscopic glass slide and was air-dried. The glass slide was fixed with 100% pre-heated glycerine and was visualised under a Zeiss light microscope at 20 X and 100 X (Whitney and Needham 2014). Pollen diameter was calculated through Image-J software (153-win-java8). Scanning electron microscopy (SEM) of dried *P. pratense* pollen was done on a HITACHI SU 1500 equipment. Pollens were directly kept on a carbon patch covered SEM stub holders. Their diameters were calculated through SEM microscale tool at 50 µm resolution at 1 KV accelerating voltage in a high vacuum mode (Depciuch et al. 2016).

Pollen Fourier transform infrared spectroscopy

Dried pollen grains (20 mg) of R1, R2 and R3 were placed on fourier transform infrared spectroscopy (FTIR) IRTracer-100 knob for FTIR analysis (Kenđel and Zimmermann 2020). Vertex 70 V FTIR spectrometer measurements were taken using the attenuated total reflectance (ATR) method. Resolution of 4 cm^{-1} was selected for infrared radiations in the range of 500–4500 cm^{-1} to draw peaks (Depciuch et al. 2018). Normalized obtained peaks and saved data files in comma-separated values (CSV) format that were processed for drawing FTIR peaks through Origin pro 8.5 E-2018 software.

Identification of pollen associated bacteria

P. pratense pollen samples from the selected regions were shaken for 30 minutes in 1 ml of 0.9% saline four-fold dilutions were made. 100 µl of the last dilution was plated on Luria Bertani (LB) agar plate in an ESCO class II biosafety cabinet (SC2-4E1). Plates were incubated for 48 hours at 33°C. Morphologically similar colonies appeared on plates from all three samples of R1, R2 and R3 regions within 24 hours. The bacterium was purified and glycerol stocks were prepared. The purified bacterial culture was subjected to identification through Biolog GEN III system (Biolog, Hayward, CA, USA) to test the bacterium ability to utilize 71 carbon sources present in the microplate. Sub-cultured single colony from 24 hours bacterial culture on Biolog Universal Growth (BUG) media. Single colony from the BUG media petri plate through sterile inoculator was taken and swirled the inoculum in inoculation fluid A (IF-A) to release bacteria colony in the IF-A. Then checked IF-A turbidity before and after the release of bacterial colony in the fluid, and ensured that the transmittance value of the IF-A was above 90%. After that, transferred 100 µl IF-A to each well of the GEN III microplate through a multichannel pipette. Incubated the plates at 33°C for 24 hours, and recorded plate readings visually from the colour readings after 6 hours and 22 hours of

incubation. Colour change in the microplate wells was compared to the negative and positive controls, well #1 and well #10 respectively. The plate 22 hours' results were used for the identification of bacterium from Biolog database.

Protein extraction

Total soluble proteins of *P. pratense* dry pollens were extracted through defatting and non-defatting methods in 1X-phosphate buffer saline (PBS) (Cases et al. 2014) with some modifications. For defatting, suspended 1 g of pollen in 20 volumes of N-Hexane for each sample and kept the samples shaking at room temperature for 2 hours, and filtered through Whatman filter paper. Defatted pollens were extracted in 1X-PBS, centrifuged and filtered through 0.45 µm pore size syringe filters. Aliquots of the filtrate were labelled, quantified and stored at -20°C. The same process for non-defatting was repeated.

Human sera selection

The allergenic human sera to *P. pratense* listed in Table 1 was purchased from Plasma Lab International (3128 Norton Avenue, Everett, WAA 98201 USA http://www.plasmalab.com. Also, *P. pratense* allergenic sera available in FARRP Lab University Of Nebraska Lincoln USA were used, and serum was collected from Pakistani student at the University of Nebraska Lincoln USA (Ramadan et al. 2021). Ethical consent from donors were taken to use their serum in research following approval by the collecting source of serum donors as required by the Institutional Review Board (IRB) of the University of Nebraska-Lincoln.

Table 1
Summary of the selected serum samples clinical history.

serum ID	Gender /Race	Reported allergies	Symptoms	ICap Total IgE	Specific IgE CAP		
					P. pratense	*Birch*	*Ragweed*
PL22241	M /Caucasian	Cat dander, dog dander, *P. pratense* pollen ragweed pollen	Allergic rhinitis and FEIA	453	7	0.6	17.4
PL22272	M /Caucasian	Ragweed, birch pollen	Allergic rhinitis and FEIA	NA	1.1	2.6	11.2
PL22887	M /Caucasian	Cat, Soy, hazelnut, almond, walnut, *P. pratense* pollen, ragweed, sunflower	Allergic rhinitis, FEIA	354	13	5.8	7.8
PL 24180-D	F /American	Dog and cat dander, *P. pratense*	Allergic rhinitis, asthma	135	10.5	1.53	0
PL 24329-I	F /Caucasian	Multiple allergies	Allergic rhinitis	378	31.8	0	0
PL 25218-G	M /American	Grass pollen	Allergic rhinitis	413	48.4	0	0
PL 29148	M /Caucasian	NA	Allergic rhinitis and asthma and FEIA	525	0	0	0
19362-CM	F /Caucasian	*A. hypogea, orchard grass, P. pratense, birch, ragweed, mugwort*	NA	377	61.6	20	31.2
23508-JK	M /Caucasian	NA	NA	300	10.4	3.42	0
PL 27121	M /Caucasian	NA	NA	4054	0	0	61

NA = Unknown information

serum ID	Gender /Race	Reported allergies	Symptoms	ICap Total IgE	Specific IgE CAP		
					P. pratense	*Birch*	*Ragweed*
22329 JE	M /American	NA	NA	*1953*	100	13.1	34
19392 CS	NA/NA	*Soy, A. hypogea*		NA	0	0	0
9735 RE	NA/NA	NA	Anaphylaxis to peanut.	NA	0	0	0
22104 JC	M /Caucasian	NA	Ragweed, house dust mite,	NA	0	0	0
PL27097	M /Caucasian	NA	*A. hypogea*, Birch, Ragweed	2692	0	87.7	18.1
PakPoln	M/ Caucasian	*P. pratense, B. papyrifera, C. sativa*	NA	NA	0	0	0
NA = Unknown information							

Allergenicity testing

The pollen protein extract's allergenicity was confirmed by IgE testing with selected sera through dot blot followed by western blot analysis (Ramadan et al. 2021) with slight modifications. Monoclonal mouse anti-human IgE conjugated with horseradish peroxidase (HRP) were used (Southern Biotech, Birmingham; AL clone B3102E8 Cat #9160–05) 1:1000 diluted in PBS-T. IgE binding was detected with Super-signal West Dura Extended Duration Chemi luminescent (ECL) substrate (Pierce, Rockford, IL, USA, Cat # 34,076) on a UVP Bio-Spectrum 815 Imaging System for 35 minutes. Manually set up image intensities to visualize and reduce the background noise.

Results

Pollen microscopy

SEM and light microscopy at 100 X found round structure of *P. pratense* pollen. Pollen exine layer has numerous spikes on it. There was a scar like structure on each pollen that attached it to the pollen tube. Average pollen diameter was 36.3 µm, 39.6 and 38 µm for R1, R2 and R3.

Figure 1 Shows *P. pratense* pollen SEM (A) and light microscopy images (B) taken at 100X resolution from three climatic regions R1, R2 and R3. The black line in the centre shows the average diameter of pollens calculated through SEM in built microscale. B shows pollens of R1, R2 and R3 regions coloured

with Ponseau S stain and captured through light microscope having circular morphology with spikes in the form of dots.

Pollens Fourier Transform Infrared Spectroscopy (FTIR)

The pollen FTIR analysis of R1, R2 and R3 regions was done using a spectral wavelength of 500–4000 nm. Proteins region and lipids spectral region, peaks analysed at 1700–1500 cm^{-1}, and (2900–2700 cm^{-1}) respectively showed variation in proteins and lipids functional group peaks (Fig. 2 and Fig. 3). Peak transmittance values and their respective spectra differed for all the three regions' pollens. Transmittance values were high for R1 having elevated mean annual temperature and low mean annual precipitation as compared to R2 and R3. Proteins and lipids functional groups spectral zones along with peak frequencies and their chemical bonds (Depciuch et al. 2017), are given in Table 2.

Table 2
Spectral zone, functional group frequencies, and associated chemical bonds

Spectral zone	Peak frequency cm^{-1}	Chemical bonds
Amide I	1620 β-sheet	NH_2 bending
1700 – 1600 cm^{-1}	1650 α-helix	N-O stretch
Amide II	1670 β-turn	
1600 – 1500 cm^{-1}	1550	
Lipids	2930	CH_3 stretching (lipids)
	2850	CH_2 stretching (lipids)

Figure 2-I Shows *P. pratense* pollen FTIR spectral peak of protein region, and Fig. 2-**II** spectral peak of lipid region. The Y-axis denotes transmittance values, and the X-axis shows wavelength. Black, red and blue colour show pollen samples of R1, R2 and R3 regions respectively. The dotted line shows spectral peak differences at a specific functional group within the protein region.

Pollen associated bacteria Biolog identification

Biolog identification system using GEN III microplate identified *B. epidermidis*. The plate has 71 carbon sources, negative and positive controls. The bacterium utilized completely 1% NaCl, 4% NaCl, 8% NaCl, 1% sodium lactate, guanidine HCl, nalidixic acid, lithium chloride, aztreonam, sodium butyrate and sodium bromate. Complete utilization of the mentioned media developed colour similar to well A10 (positive control). The bacteria completely failed to utilize D-raffinose, D-sorbitol, pectin, α-D-lactose, D-maltose, D-melibiose, L-galactonic acid lactone, D-trehalose, β-methyl-D-glucoside, D-cellobiose, D-salicin, sucrose, N-acetyl-β-D-mannosamine, D-turanose, N-acetyl-β-D-galactosamine, D-aspartic acid, stachyose, D-serine, troleandomycin, lincomycin, vancomycin, fusidic acid, rifamysin SV, tetrazolium violet, D-serine, minocycline, niaproof 4 and tetrazolium blue. Complete failure to utilize the media gave no colour similar

to well A1 (negative control). The bacterium utilized remaining 71 carbon sources partially and developed light purple colour. The dark purple-coloured wells showed strong catalase positive reaction, light coloured wells depicted slight catalase positive, and no colour development showed oxidase negative characters of the plate. Comparison of the nutrient utilization and the colour development pattern identified the bacteria as *B. epidermidis.*

Pollen protein extraction

Total pollen protein extracted in 1X PBS through defatting and non-defatting shows no significant difference in the two extraction methods. Protein quantities detected through non-defatting and defatting methods were (2.3 µg and 3.642 µg), (8.024 µg and 8.3 µg) and (6.6 µg and 7.1 µg) for R1, R2 and R3 respectively. R1 has the lowest protein quantity, while R2 has the highest protein quantity.

Dot Blot Analysis

Pollen protein extracts serum IgE binding results through dot blot showed allergenicity towards 16 human sera (see Fig. 4). Sera having strong IgE binding showed intense reaction and large diameter dots at 3 minutes exposure under UVP imager for pure protein extracts and their 1:10 dilution in 1X PBS. Sera having weak IgE binding showed faint reaction and small dot diameter under UVP imager. Less IgE reactive sera showed IgE binding in dot blots only for pure protein and did not show IgE binding with diluted protein extracts.

Figure 3P. *pratense* pollen-protein dot blot analysis. The dot in the box shows pollen allergen reactivity with primary antibody present in the serum of study participants. The bigger dots show pure pollen protein dot, while the smaller dot denotes 1/10 1X PBS diluted protein. The number above the box represents the serum identification number used in the study.

SDS-PAGE pollen proteins profile

R1, R2 and R3 pollen protein extracts resolved on Invitrogen 10–20% tris glycine premade gel showed differential banding pattern at non-denaturing and denaturing conditions. R1 having less protein concentrations showed fewer and faint bands at non-denaturing and denaturing conditions. R2 and R3 showed similar banding pattern at non-denaturing and denaturing conditions.

Figure 4 Invitrogen 10–20% tris glycine premade gel run for 90 minutes of *P. pratense* pollen protein under 130 volt constant voltage. *P. pratense* defatted pollen protein (4 µg /15µl) extracted in 1X PBS were loaded in each well. Lane 1 is precision plus protein marker, lane 2 is *P. pratense* Peshawar 2022, lane 3 is *P. pratense* Islamabad 2022, lane 4 is *P. pratense* Kotli 2022 loaded at 25°C under non-reducing conditions. Lanes 5, 7 and 9 are blank. Lane 6 is *P. pratense* Peshawar 2022, lane 8 is *P. pratense* Islamabad 2022, and lane 10 is *P. pratense* Kotli 2022, loaded at 95°C and under reducing conditions.

Western Blot Analysis

Pollen protein extracts in 1X PBS were loaded (4 µg /15µl) to each well, SDS PAGE was run, and proteins were transferred to NC membrane at a constant voltage of 30 volts and run for 90 minutes. Primary

antibody, (300 µl serum samples) were diluted into (1:10). The secondary antibody (200 µl), HRP mouse conjugated antihuman IgE Southern Biotech was diluted into (1:1000). NC membrane showed allergen bands under UVP imager. PL 22287 serum showed about 32 kDa bands putative Phl p 1 allergen for R2 and R3 (Fig. 5-I). For Pak Poln serum and 26847, proteins transferred to NC membrane showed putative allergen, either Phl p 2 or Phl p 3 for R2 and R3 (Fig. 5-II, and Fig. 5-III). For 9735 RE serum, proteins transferred to NC membrane showed putative allergen, either Phl p 2 or Phl p 3 for R2 and R3. R 3 showed two extra bands of about 14 kDa and 32 kDa. These bands show putative Phl P 1 or Phl p 5 (Fig. 5-IV).

Figure 5-I, **II, III and IV** *P. pratense*-pollen western blotting results for serum samples PL 22287, Pak Poln, 26847 and 9735 RE respectively (Table 1). A shows protein transfer to NC, B shows allergens on NC visualised under UVP Imager at 3 minutes exposure while C shows inverted image of B. M is precision plus protein marker. Lanes 1, 2 and 3 are R1, R2 and R3 pollens proteins loaded at 25°C non-reducing conditions.

Proteins transferred to NC membrane showed bands for R2 and R3. 27121 RE serum (300 µl) used as primary antibody, and diluted 1:10. The secondary antibody, HRP mouse conjugated antihuman IgE Southern Biotech 1:1000 diluted showed about 10 kDa bands for R2 and R3, and R2 showed additional allergens about 12 kDa and 14 kDa at 3 minutes exposure under UVP imager. The same NC membrane showed about 55 kDa allergen Phl p 4 for R2 and R3 samples.

Figure 6P. *pratense*-pollen western blotting results for serum 27121 (Table 1). Pollen protein extracts in 1X PBS were loaded (4 µg /15µl) to each well, SDS PAGE was run, and proteins were transferred to nitrocellulose membrane at a constant voltage of 30 volts and run for 90 minutes. A shows protein transfer to NC, B shows allergens on NC visualised under UVP Imager at 3 minutes exposure while C shows inverted image of B. D shows the same western blot visualized under 5 minutes exposure while E is its inverted image of D. M is precision plus protein marker. Lanes-2, 3 and 4 are R1, R2 and R3 pollens proteins loaded at 25°C non-reducing conditions.

Discussion

Pollens can easily come across the nasal epithelial cavity during breathing, where allergenic pollens cause allergic reactions. Pollen based allergies are increasing with climate change due to pollen season extension, increase in pollen amount, and change in allergen concentration (Ziska 2021). Climatic conditions have also pronounced effect on pollen morphological and biochemical features, and protein expression profiling. *P. pratense* is an abundant perennial grass that produces allergenic pollens. In this study, we have investigated the effect of an increase in mean annual temperature and decrease in mean annual precipitation in three different regions (R1, R2 and R3) on *P. pratense* pollen biochemical composition, and beta-expansin and profilin expression which has potential role in pollen growth and development. The mean pollen diameter of the R1 region (42.73 µm) was smaller than R2 (44.77 µm) and R3 (45.38 µm). R1 region has increased mean annual temperature and decreased mean annual precipitation in comparison to R2 and R3 regions. In previous investigations (Jung et al. 2021), it was

observed that decrease in mean pollen diameter (lower size & weight) of *B. pratense* was in response to drought stress. There were no other significant differences in pollen grain structural morphology, and the microscopic identification of pollen cannot differentiate pollens within a specie (Radaeski et al. 2016).

Water soluble proteins content were lower in R1 region pollens in comparison to R2 and R3 that is similar to previous findings that *P. pratense* pollen has lower protein and allergen content under drought stress conditions (Jung et al. 2021). It was observed that 0.87°C warming treatment according to representative concentration pathway (RCP) 2.6, yielded 2.4 ng protein and 0.029 ng allergen/per grain in comparison to the control group 3.1 ng protein and 0.040 ng allergen/per grain. Under drought stress conditions, *P. pratense* pollens showed lower size and weight under drought stress condition. Our results showed that R1 (drought stress conditions) pollen protein concentration was 3.6 µg/10 µl, in comparison to R2 and R3 (7.1 µg/10 µl and 8.3 µg/10 µl) respectively. Overall, differences in pollen protein concentration, associate to variation in environmental conditions of different regions. We observed an increased mean annual temperature and decreased mean annual precipitation in R1 region in comparison to R2 and R3. The diameter of *P. pratense* pollens of R1 region was smaller (42.73 µm) in comparison to R2 (44.77 µm) and R3 region (45.38 µm) (see Fig. 1). We could not see any other significant differences in pollen grain structural morphology.

FTIR analysis showed differences in pollens biochemical profile as the spectral peaks differ in pollens of all three regions (see Fig. 2-I and Fig. 2-II). Region R1 has elevated mean annual temperature and low mean annual precipitation but lower elevation from sea level in comparison to R2 and R3. Therefore, FTIR spectral peaks reflected that R1 pollens transmittance values were high in comparison to R2 and R3 regions. R2 and R3 regions have almost similar temperature and precipitation conditions but differ in elevation. However, the pollens of these two regions showed similar spectral peaks. Spectral differences of pollens growing in different environmental conditions correlate to differences in pollen protein, carbohydrate, lipids and sporopollenin (Zimmermann and Kohler 2014). They have studied 300 different plants species pollens collected from variant environmental regions and found differences in pollen FTIR peaks within single species pollen collected from different environmental regions. Our results indicated that spectral bands differ at 1540 cm^{-1} and 1670 cm^{-1} respectively for R1, R2 and R3 (Fig. 2-I). Spectral bands at 1540 cm^{-1} and 1670 cm^{-1} correspond to amide-II and amide-I respectively (Diehn et al. 2020). Similarly, 2850 cm^{-1} and 2930 cm^{-1} spectral bands correspond to lipids, and the spectral bands differ at these wavelengths for R1, R2 and R3. These differences in pollen FTIR peaks associated with different regions environmental conditions. Environment interactions influence pollen biochemical composition and pollen phenotypic plasticity occurred due to locational environment (Bagcoglu et al. 2017) causing variations in pollen proteins, lipids, sporopollenins and carbohydrates. Moreover, pollen associated bacteria may influence pollen phenotypic plasticity.

We isolated gram positive, catalase positive and oxidase negative bacterium from *P. pratense* pollen, having optimal growth at 33°C. The bacterium formed grey white colonies on LB agar for 24 hours of incubation period. Biolog Gen III plate testing identified the bacterium as *B. epidermidis*. We found no bacterial diversity on the pollen collected from three different regions. *B. epidermidis* was discovered from

skin sample that formed 2–4 mm grey white colonies on nutrient agar media after 24 hours incubation between 30°C – 37°C. The bacterium was characterized as catalase positive, oxidase and urease negative, non-acid fast, rod shaped or coccoid and non-motile bacteria (Collins et al. 1983). Brevibacteria species are opportunistic pathogens mostly isolated from clinical samples (Wauters et al. 2004). *Staphylococcus epidermidis* and *B. epidermidis* are classified as risk group II and risk group I respectively that cause negative pulmonary health effects (Martin et al. 2010). Comparisons of allergenic and non-allergenic plants pollens and pollens associated bacterial endotoxin analysis showed that pollen related microbiota influence pollen allergic potential (Cardinale et al. 2019). Similar to our findings, only *Bacillus* species was identified from *P. pratense* pollen sampled from 20 different urban and rural sites (Obersteiner et al. 2016). These findings strengthen our hypothesis that pollen associated microbes may play an important role in pollen allergies. Allergenic pollen associated microbiome role should be studied further to explore pollen allergies. Furthermore, pollen allergen expression in respect to locational environment is also important for *P. pratense*-pollen allergies.

The pollen protein extracts from all regions resolved on 10% tris glycine gels found similar protein banding pattern for R2 and R3 regions. However, the R1 banding pattern was different and lower than R2 and R3 regions pollens. R2 and R3 have almost similar temperature and precipitation conditions but different elevations. R1 has different temperature, precipitation and elevation in comparison to R2 and R3. Increase in temperature and precipitation induces pollen production in early flowering species (Makra et al. 2014). *P. pratense* is early flowering specie but nonavailability of pollen count data from all regions limits the study to analyse pollen production timings. Similarly, it has been found that *Poaceae* flowering phenology and pollen count is affected by rainfall, maximum temperature, wind speed and humidity (Norris-Hill 1997; Jato et al. 2009). Rainfall affects total pollen count as compared to temperature (Jato et al. 2009). Air temperature facilitates anther opening, pollen release and pollen dispersal (Ščevková et al. 2020). The difference in environmental conditions in the three regions have caused differences in the PBS soluble protein expression. Water stress, heat stress and air pollution induces infertility in pollen (Shyam S. Mohapatra 1996). R1 has about 1.5°C and 1.0°C higher temperature R2 and R3 respectively (Humayun et al. 2023). Pollen tube growth and development is sensitive to temperature stress. Heat shock proteins (HSPs) are active in immature pollen in the absence of heat stress. Response of HSPs decrease in mature pollen due to heat stress (Mascarenhas and Crone 1996). The decrease in *P. pratense*-pollen weight, and protein content growing under drought and height conditions is reported (Jung et al. 2021). They have found significantly increased allergen content in control treatments as compared to heat treatments (warming treatment, warming + drought treatment). Our study found that putative allergens' expression in *P. pratense* pollen has also changed with the change in climatic conditions in the three regions.

Decrease in protein quantity and intensity has caused low or no expression of putative allergens in R1 in comparison to R2 and R3 regions for several *P. pratense*-pollen allergens. Phl p 1 (32 kDa allergen) is a beta expansin involved in cell wall loosening during pollen tube growth (Sampedro and Cosgrove 2005). Phl p1 allergen expressed in R2 and R3, while its expression was missing in R1. Environmental conditions difference of R1 as compared to R2 and R3 have affected on Phl p 1 allergen (see Fig. 5-I). Similarly, R1

and R3 have different mean annual precipitation and elevation from R2. Lol p 1 allergen, (27 kDa beta-expansin) is a major Lolium perenne allergen, and a member of *Poaceae* family (Griffith et al. 1991). Lol p 1 differential expression was found in patients living in temperate and sub-tropic regions. Lol p 1 differential expression had occurred in allergic rhinitis participants in different biogeographical regions {temperate regions (latitude 35°S, 30.8°C) and sub-tropic regions (latitudes 21 to 27°S, 27.6°C)} (Kailaivasan et al. 2020). In our findings beta-expansin allergen expression in pollen reduces or diminishes with increase in temperature. Similarly, Fig. 5-II, Fig. 5-III and Fig. 6 denote Phl p 2/ Phl p 3 which are a 10.9 and 12 kDa pollen allergens (Dolecek et al. 1993; Petersen et al. 2006). These allergens contain a C-terminal expansin domain, and their sequence homology shows common origin of evolution with Phl p 1 (Petersen et al. 2006). Phl p 2 and Phl p 3 expression differs in *P. pratense* pollen studied in the three regions. Phl p 2 and Phl p 3 expression is missing in R1 in western blot at 3 minutes exposure in UVP imager 8.5 while western blot 5 minutes exposure showed very slight expression of Phl p 2 in the R1 region. R1 has elevated temperature (23.06°C) and decreased precipitation (42.31 mm) conditions as compared to R2 (22.07°C, 102.445 mm) and R3 (21.39°C, 110.34 mm) having low temperature and increased precipitation conditions. On molecular level, increasing temperature accumulates reactive oxygen species (ROS) and their role is important in pollen tube growth and cause pollen infertility (Pacini and Dolferus 2019; Santiago and Sharkey 2019). Proline amino acid levels decrease under heat stress conditions (Santiago and Sharkey 2019) that strengthen our findings where elevated temperature at R1 has reduced allergen expression. Similarly, the western blot 5 minutes exposure in UVP imager 8.5 showed very faint expression of Phl p 4 in R2 and R3 samples (see Fig. 6). Phl p 4 (55 kDa berberine bridge like-enzyme) occurs in exine, cytoplasm and amyloplast of pollen grain, and involved in the biosynthesis of sporopollenin (Fischer et al. 1996; Visez et al. 2021). It is a glycosylated protein and is a crossreactive carbohydrate determinant which produce low antibodies consequence to sensitization (Westman et al. 2020). Our results showed that increase in temperature has diminished or minimized the expression of Phl p 4. Similarly, Phl p 5 is a major and 32 kDa allergen. Till date, the allergen is not fully characterized and its role in pollen development and pollen release needs to be explored. Our results found that Phl p 5 expression was absent in R1 as compared to R2 and R3 (see Fig. 6). The study findings suggest that increase in air temperature and decrease in precipitation inhibit expression of the putative allergen in R1. Phl p 12 (14 kDa profilin) allergen is a ubiquitous pollen protein found in pollen grains of many monocot and dicot plants. The protein carries a high degree of sequence homology with other plant profilin (Valenta et al. 1994). Profilin regulates actin binding and cell signalling during cell division in pollen tube development (Jimenez-Lopez et al. 2012; Liu et al. 2021). We show that the allergenic profilin expression occurs in R2, and is missing in R1 and R3 region pollens. Differences in environmental conditions between the three regions affect the expression of Phl p 12. Increase in temperature and decrease in precipitation inhibit the putative allergen expression that suggest reduction of associated allergies.

Putative allergens Phl p 2 or Phl p 6 expressed in R2 and R3 only (see Fig. 5-IV). Additionally, Phl p 12 and Phl p 5 showed expression in R3 only. Phl p 12 is a profilin that expressed in R3, while Phl p 5 is an unknown allergen. Our findings suggest that Phl p 1 a cell wall protein (Hoffmann et al. 2022) is a major

P. pratense pollen allergen (García-Mozo 2017) expression occurs in R2 and R3. Sensitization to *P. pratense* depends on the immunity level of a subject, allergen exposure time and allergen cross reactivity. Several allergens of grass pollen cause oral allergy syndrome (OAS) where allergy is induced by exposure of a subject to pollen allergens and food allergens (García-Mozo 2017). Such type of allergy due to allergen cross reactivity is caused by minor allergens like polcalcins, profilins and lipid transfer proteins (Radauer and Breiteneder 2006).

Conclusion

The findings of this study elaborate that difference in climatic conditions affect *P. pratense* pollen size, biochemical composition and allergen expression. R1 region yielded small *P. pratense*-pollen in comparison to R2 and R3 region. FTIR spectral peaks varied for R1, R2 and R3 pollen that marked difference in pollen biochemical composition. We report for the first time *B. epidermidis* from *P. pratense* pollen through Biolog identification system that may have a significant role in *P. pratense*-pollen allergies which need to be further investigated. SDS PAGE showed loss of certain protein bands from the R1 region in comparison to R2 and R3. Similarly, western blot analysis showed that putative allergens-Phl p 1, Phl p 2 and Phl p 5 were absent in R1 pollen proteins, and were present in R2 and R3 pollen proteins. The findings suggest that increase in temperature up to 1.5°C and decrease in precipitation inhibit expression of allergens during pollen development.

Declarations

Author statement

MH performed the experiment, compiled the data and wrote the manuscript. SN helped in project execution, data compilation and analysis and wrote the manuscript. RG helped with experimental design, co-supervised the work, reviewed the manuscript, and improved the English language of the manuscript. ZA conceptualized the idea, designed the experimental strategies, reviewed the manuscript and supervised the whole project.

Acknowledgments

We are thankful to the Higher Education Commission (HEC) Pakistan for providing financial support to conduct this study under the umbrella of HEC-NRPU Project No. 8231 titled "Strategic development of baseline protocols to predict climate change impacts on plants and prevalence of associated allergies in Pakistan". We are also thankful to Climate Data Processing Centre, Pakistan Meteorological Department, Pakistan, and Meteoblue, University of Basel, Switzerland for providing precipitation and temperature data.

The authors declare no conflict of interest.

References

1. Akdis CA (2021) Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? Nat Rev Immunol 21:739–751. https://doi.org/10.1038/s41577-021-00538-7
2. Bagcoglu M, Kohler A, Seifert S, et al (2017) Monitoring of plant environment interactions by high-throughput FTIR spectroscopy of.pdf. Methods Ecol Evol 8:870–880
3. Beggs PJ (2021) Climate change, aeroallergens, and the aeroexposome. Environ Res Lett 16:. https://doi.org/10.1088/1748-9326/abda6f
4. Cardinale M, Maisinger C, Schnell S (2019) THE MICROBIOME OF FLOWER POLLEN AND ITS POTENTIAL IMPACT ON POLLEN-RELATED ALLERGIES BINOY. 72:65–72
5. Cases B, Ibañez MD, Tudela JI, et al (2014) Immunological cross-reactivity between olive and grass pollen: Implication of major and minor allergens. World Allergy Organ J 7:1–7. https://doi.org/10.1186/1939-4551-7-11
6. Cavuoto KM, Zhu AY (2022) The Role of the Ocular Surface Microbiome (OSM) in Diseases of the Anterior Segment and Ocular Surface. Curr Ophthalmol Rep 10:179–187. https://doi.org/10.1007/s40135-022-00294-4
7. Collins MD, Farrow JAE, Goodfellow M, Minnikin DE (1983) Brevibacterium casei sp. nov. and Brevibacterium epidermidis sp. nov. Syst Appl Microbiol 4:388–395. https://doi.org/10.1016/S0723-2020(83)80023-X
8. Depciuch J, Kasprzyk I, Drzymała E (2018) Identification of birch pollen species using FTIR spectroscopy. Aerobiologia 34:525–538. https://doi.org/10.1007/s10453-018-9528-4
9. Depciuch J, Kasprzyk I, Sadik O, Parlińska-Wojtan M (2017) FTIR analysis of molecular composition changes in hazel pollen from unpolluted and urbanized areas. Aerobiologia 33:1–12. https://doi.org/10.1007/s10453-016-9445-3
10. Depciuch, Kasprzyk I, Roga E, Parlinska-Wojtan M (2016) Analysis of morphological and molecular composition changes in allergenic Artemisia vulgaris L. pollen under traffic pollution using SEM and FTIR spectroscopy. Environ Sci Pollut Res 23:23203–23214. https://doi.org/10.1007/s11356-016-7554-8
11. Diehn S, Zimmermann B, Tafintseva V, et al (2020) Combining Chemical Information From Grass Pollen in Multimodal Characterization. Front Plant Sci 10:1–18. https://doi.org/10.3389/fpls.2019.01788
12. Dolecek C, Vrtala S, Laffer S, et al (1993) Molecular characterization of Phl p II, a major timothy grass (Phleum pratense) pollen allergen. FEBS Lett 335:299–304. https://doi.org/10.1016/0014-5793(93)80406-K
13. Fischer S, Grote M, Fahlbusch B, et al (1996) Characterization of Phl p 4, a major timothy grass (Phleum pratense) pollen allergen. J Allergy Clin Immunol 98:189–198. https://doi.org/10.1016/S0091-6749(96)70242-7
14. García-Mozo H (2017) Poaceae pollen as the leading aeroallergen worldwide: A review. Allergy Eur J Allergy Clin Immunol 72:1849–1858. https://doi.org/10.1111/all.13210

15. Griffith IJ, Smith(xa2) PM, Pollock J, et al (1991) Cloning sequencing of Lol pI, the major allergenic protein of rye-grass pollen. FEBS Lett 279:210–215. https://doi.org/10.1016/0014-5793(91)80151-R
16. Hoffmann K, Hilger C, Santos A, Dramburg S (2022) Molecular Allergology User's Guide 2.0
17. Humayun M, Naseem S, Goodman RE, Ali Z (2023) Climate Change Impacts on Broussenetia papyrifera Pollens - Metabolome Investigations and Prospects of Allergy Prevalence. Aerobiologia 92:1–27
18. Jato V, Rodríguez-Rajo FJ, Seijo MC, Aira MJ (2009) Poaceae pollen in Galicia (N.W. Spain): Characterisation and recent trends in atmospheric pollen season. Int J Biometeorol 53:333–344. https://doi.org/10.1007/s00484-009-0220-9
19. Jimenez-Lopez JC, Morales S, Castro AJ, et al (2012) Characterization of profilin polymorphism in pollen with a focus on multifunctionality. PLoS ONE 7:. https://doi.org/10.1371/journal.pone.0030878
20. Jung S, Estrella N, Pfaffl MW, et al (2021) Impact of elevated air temperature and drought on pollen characteristics of major agricultural grass species. PLoS ONE 16:1–19. https://doi.org/10.1371/journal.pone.0248759
21. Kailaivasan TH, Timbrell VL, Solley G, et al (2020) Biogeographical variation in specific IgE recognition of temperate and subtropical grass pollen allergens in allergic rhinitis patients. Clin Transl Immunol 9:1–11. https://doi.org/10.1002/cti2.1103
22. Kenđel A, Zimmermann B (2020) Chemical Analysis of Pollen by FT-Raman and FTIR Spectroscopies. Front Plant Sci 11:1–19. https://doi.org/10.3389/fpls.2020.00352
23. Liu C, Zhang Y, Ren H (2021) Profilin promotes formin-mediated actin filament assembly and vesicle transport during polarity formation in pollen. Plant Cell 33:1252–1267. https://doi.org/10.1093/plcell/koab027
24. Lucas JA, Gutierrez-Albanchez E, Alfaya T, et al (2019) Oxidative stress in ryegrass growing under different air pollution levels and its likely effects on pollen allergenicity. Plant Physiol Biochem 135:331–340. https://doi.org/10.1016/j.plaphy.2018.12.018
25. Makra L, Csepe Z, Matyasovszky I, et al (2014) Interdiurnal Variability of Artemisia, Betula and Poaceae Pollen Counts and Their Association With Meteorological Parameters. Carpathian J Earth Environ Sci 9:207–220
26. Martin E, Kämpfer P, Jäckel U (2010) Quantification and identification of culturable airborne bacteria from duck houses. Ann Occup Hyg 54:217–227. https://doi.org/10.1093/annhyg/mep088
27. Mascarenhas JP, Crone DE (1996) Pollen and the heat shock response. Sex Plant Reprod 9:370–374. https://doi.org/10.1007/BF02441959
28. Mendy A, Zeldin DC (2020) Phl p 4: An early indicator of grass pollen allergy? J Allergy Clin Immunol 145:1556–1557. https://doi.org/10.1016/j.jaci.2020.04.011
29. Norris-Hill J (1997) The influence of ambient temperature on the abundance of Poaceae pollen. Aerobiologia 13:91–97. https://doi.org/10.1007/BF02694424

30. Obersteiner A, Gilles S, Frank U, et al (2016) Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. PLoS ONE 11:1–16. https://doi.org/10.1371/journal.pone.0149545
31. Pacini E, Dolferus R (2019) Pollen developmental arrest: Maintaining pollen fertility in a world with a changing climate. Front Plant Sci 10:1–15. https://doi.org/10.3389/fpls.2019.00679
32. Petersen A, Suck R, Lindner B, et al (2006) Phl p 3: Structural and immunological characterization of a major allergen of timothy grass pollen. Clin Exp Allergy 36:840–849. https://doi.org/10.1111/j.1365-2222.2006.02505.x
33. Radaeski JN, Bauermann SG, Pereira AB (2016) Poaceae pollen from Southern Brazil: Distinguishing grasslands (campos) from forests by analyzing a diverse range of poaceae species. Front Plant Sci 7:. https://doi.org/10.3389/fpls.2016.01833
34. Radauer C, Breiteneder H (2006) Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. J Allergy Clin Immunol 117:141–147. https://doi.org/10.1016/j.jaci.2005.09.010
35. Ramadan S, Marsh J, El-Sherbeny GA, et al (2021) Purification of soybean cupins and comparison of IgE binding with peanut allergens in a population of allergic subjects. Food Chem Toxicol 147:111866. https://doi.org/10.1016/j.fct.2020.111866
36. Sampedro J, Cosgrove DJ (2005) The expansin superfamily. Genome Biol 6:1–11. https://doi.org/10.1186/gb-2005-6-12-242
37. Santiago JP, Sharkey TD (2019) Pollen development at high temperature and role of carbon and nitrogen metabolites. Plant Cell Environ 42:2759–2775. https://doi.org/10.1111/pce.13576
38. Sapkota A, Dong Y, Li L, et al (2020) Association between Changes in Timing of Spring Onset and Asthma Hospitalization in Maryland. JAMA Netw Open 3:1–11. https://doi.org/10.1001/jamanetworkopen.2020.7551
39. Sapkota A, Murtugudde R, Curriero FC, et al (2019) Associations between alteration in plant phenology and hay fever prevalence among US adults: Implication for changing climate. PLoS ONE 14:1–10. https://doi.org/10.1371/journal.pone.0212010
40. Ščevková J, Vašková Z, Sepšiová R, et al (2020) Relationship between Poaceae pollen and Phl p 5 allergen concentrations and the impact of weather variables and air pollutants on their levels in the atmosphere. Heliyon 6:. https://doi.org/10.1016/j.heliyon.2020.e04421
41. Sekerkova A, Polackova M, Striz I (2012) Detection of Phl p 1, Phl p 5, Phl p 7 and Phl p 12 Specific IgE antibodies in the sera of children and adult patients allergic to Phleum pollen. Allergol Int 61:339–346. https://doi.org/10.2332/allergolint.11-OA-0372
42. Shyam S. Mohapatra R bruce K (1996) Pollen Biotechnology Gene Expression and Characterization, 1st edn.
43. Singh N, Diebold Y, Sahu SK, Leonardi A (2022) Epithelial barrier dysfunction in ocular allergy. Allergy Eur J Allergy Clin Immunol 77:1360–1372. https://doi.org/10.1111/all.15174

44. Subiza J, Cabrera M, M CRJ, et al (2019) Influence of climate change on pollen counts and pollinosis in Madrid , a study over 40 years . p 78
45. Valenta R, Ball T, Vrtala S, et al (1994) cDNA cloning and expression of timothy grass (phleum pratense) pollen profilin in Escherichia coli: Comparison with birch pollen profilin. Biochem. Biophys. Res. Commun. 199:106–118
46. Visez N, de Nadaï P, Choël M, et al (2021) Biochemical composition of Phleum pratense pollen grains: A review. Mol. Immunol. 136:98–109
47. Wauters G, Haase G, Avesani V, et al (2004) Identification of a novel Brevibacterium species isolated from humans and description of Brevibacterium sanguinis sp. nov. J Clin Microbiol 42:2829–2832. https://doi.org/10.1128/JCM.42.6.2829-2832.2004
48. Westman M, Åberg K, Apostolovic D, et al (2020) Sensitization to grass pollen allergen molecules in a birth cohort—natural Phl p 4 as an early indicator of grass pollen allergy. J Allergy Clin Immunol 145:1174-1181.e6. https://doi.org/10.1016/j.jaci.2020.01.006
49. Whitney BS, Needham T (2014) Isopropyl alcohol: A replacement for tertiary-butyl alcohol in pollen preparations. Rev Palaeobot Palynol 203:9–11. https://doi.org/10.1016/j.revpalbo.2013.11.004
50. Xu Y, Guan K, Sha L, et al (2021) Sensitization profiles of timothy grass pollen in northern China. J Asthma Allergy 14:1431–1439. https://doi.org/10.2147/JAA.S334183
51. Zimmermann B, Bağcıoğlu M, Tafinstseva V, et al (2017) Original Research A high- throughput FTIR spectroscopy approach to assess adaptive variation in the chemical composition of pollen. 10839–10849. https://doi.org/10.1002/ece3.3619
52. Zimmermann B, Kohler A (2014) Infrared spectroscopy of pollen identifies plant species and genus as well as environmental conditions. PLoS ONE 9:. https://doi.org/10.1371/journal.pone.0095417
53. Ziska LH (2021) Climate, Carbon Dioxide, and Plant-Based Aero-Allergens: A Deeper Botanical Perspective. Front Allergy 2:1–6. https://doi.org/10.3389/falgy.2021.714724

Figures

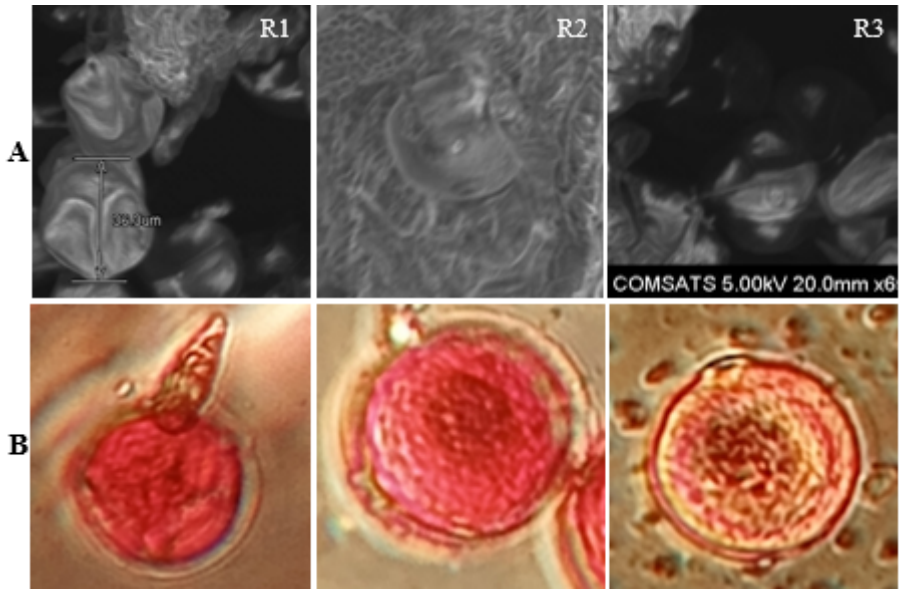

Figure 1

Shows *P. pratense* pollen SEM (A) and light microscopy images (B) taken at 100X resolution from three climatic regions R1, R2 and R3. The black line in the centre shows the average diameter of pollens calculated through SEM in built microscale. B shows pollens of R1, R2 and R3 regions coloured with Ponseau S stain and captured through light microscope having circular morphology with spikes in the form of dots.

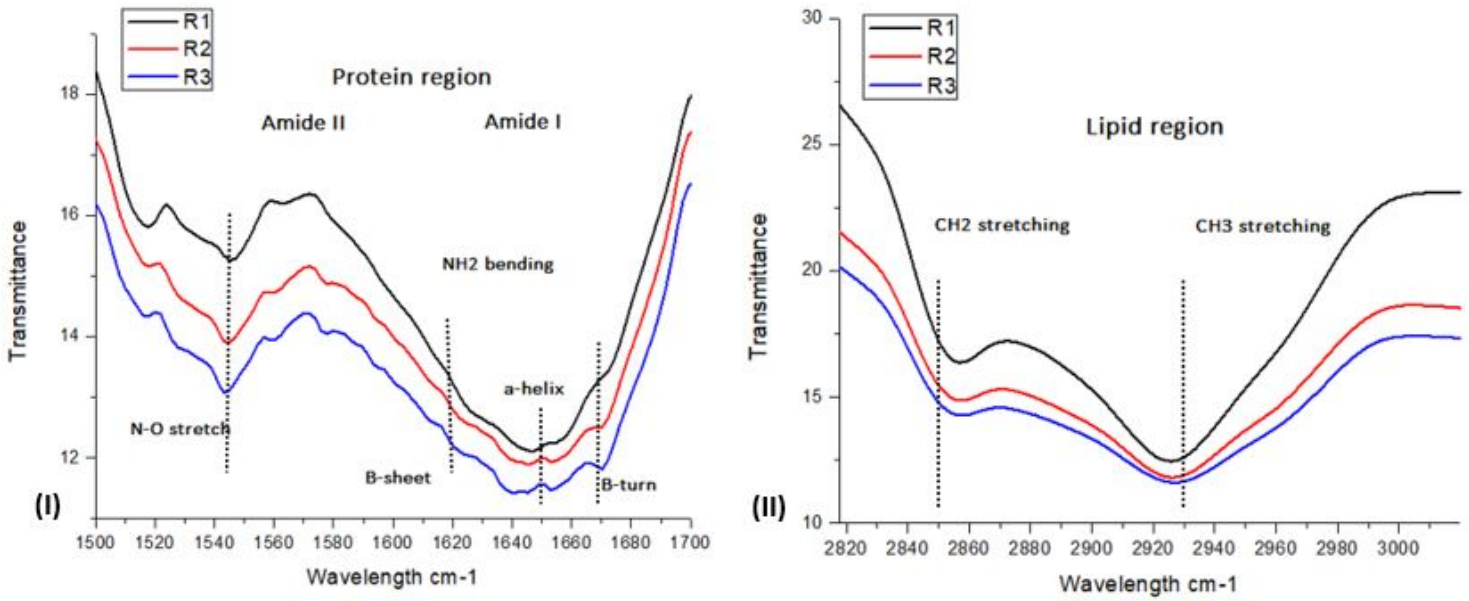

Figure 2

2-I Shows *P. pratense* pollen FTIR spectral peak of protein region, and **Fig. 2-II** spectral peak of lipid region. The Y-axis denotes transmittance values, and the X-axis shows wavelength. Black, red and blue

colour show pollen samples of R1, R2 and R3 regions respectively. The dotted line shows spectral peak differences at a specific functional group within the protein region.

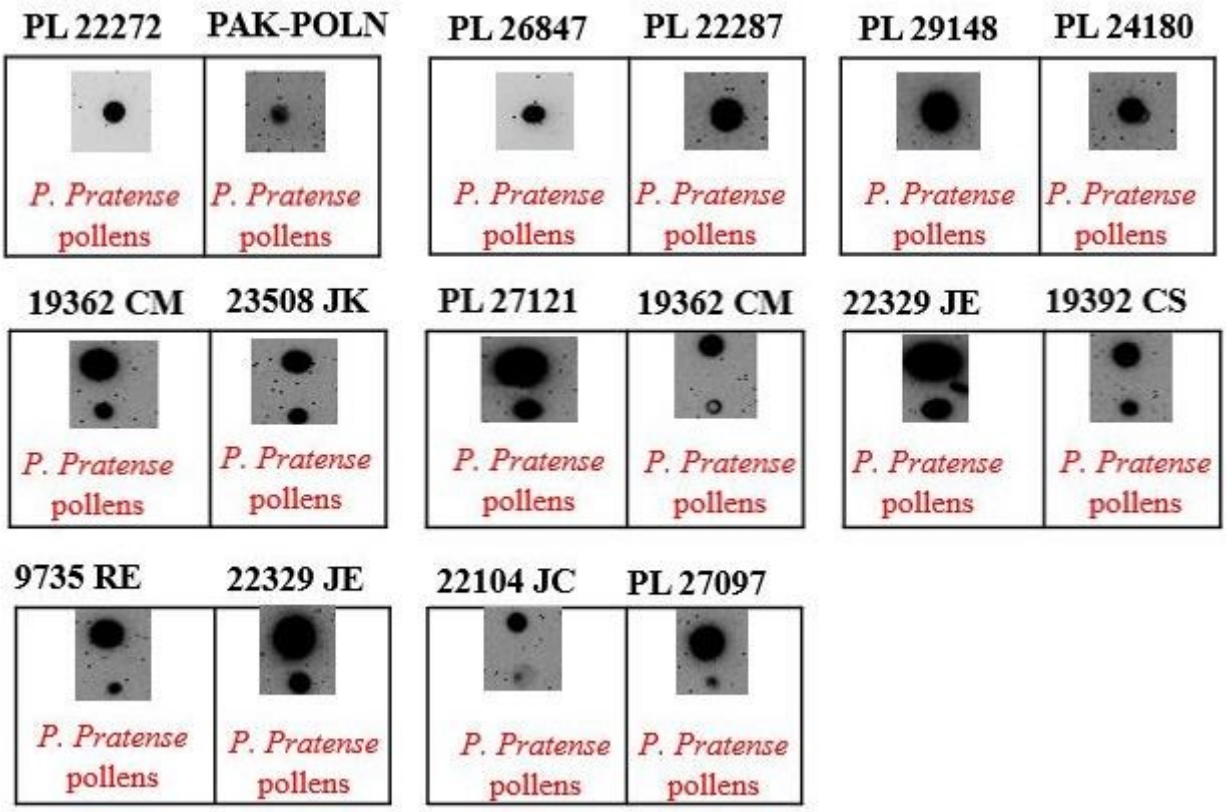

Figure 3

P. pratense pollen-protein dot blot analysis. The dot in the box shows pollen allergen reactivity with primary antibody present in the serum of study participants. The bigger dots show pure pollen protein dot, while the smaller dot denotes 1/10 1X PBS diluted protein. The number above the box represents the serum identification number used in the study.

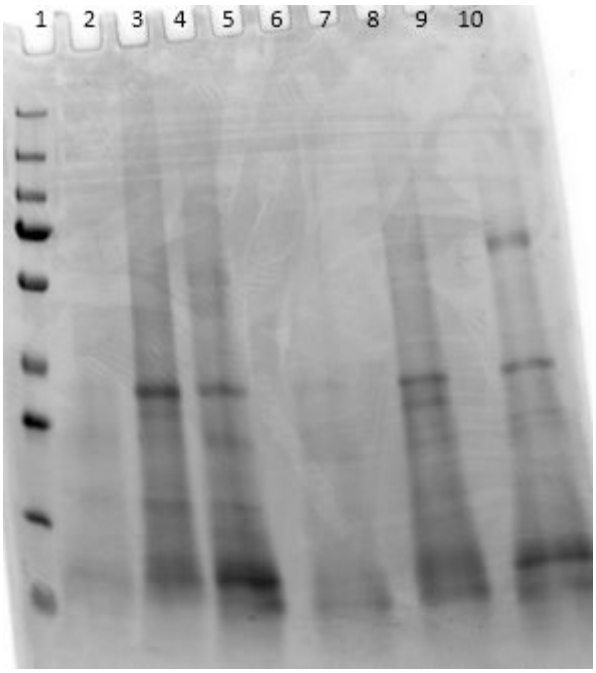

Figure 4

Invitrogen 10-20% tris glycine premade gel run for 90 minutes of *P. pratense* pollen protein under 130 volt constant voltage. *P. pratense* defatted pollen protein (4 µg /15µl) extracted in 1X PBS were loaded in each well. Lane 1 is precision plus protein marker, lane 2 is *P. pratense* Peshawar 2022, lane 3 is *P. pratense* Islamabad 2022, lane 4 is *P. pratense* Kotli 2022 loaded at 25 °C under non-reducing conditions. Lanes 5, 7 and 9 are blank. Lane 6 is *P. pratense* Peshawar 2022, lane 8 is *P. pratense* Islamabad 2022, and lane 10 is *P. pratense* Kotli 2022, loaded at 95 °C and under reducing conditions.

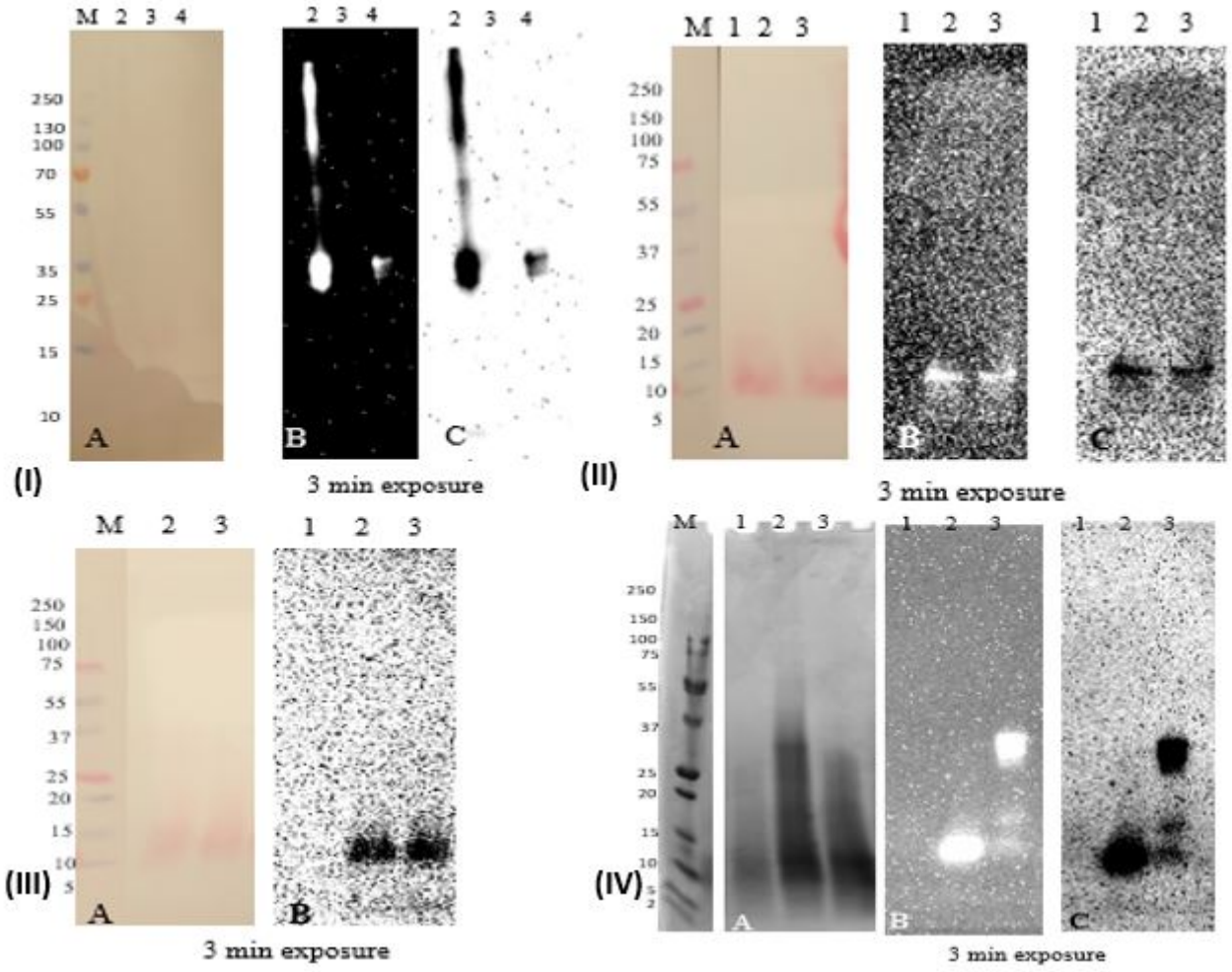

Figure 5

5-I, II, III and IV *P. pratense*-pollen western blotting results for serum samples PL 22287, Pak Poln, 26847 and 9735 RE respectively (Table 1). A shows protein transfer to NC, B shows allergens on NC visualised under UVP Imager at 3 minutes exposure while C shows inverted image of B. M is precision plus protein marker. Lanes 1, 2 and 3 are R1, R2 and R3 pollens proteins loaded at 25 °C non-reducing conditions.

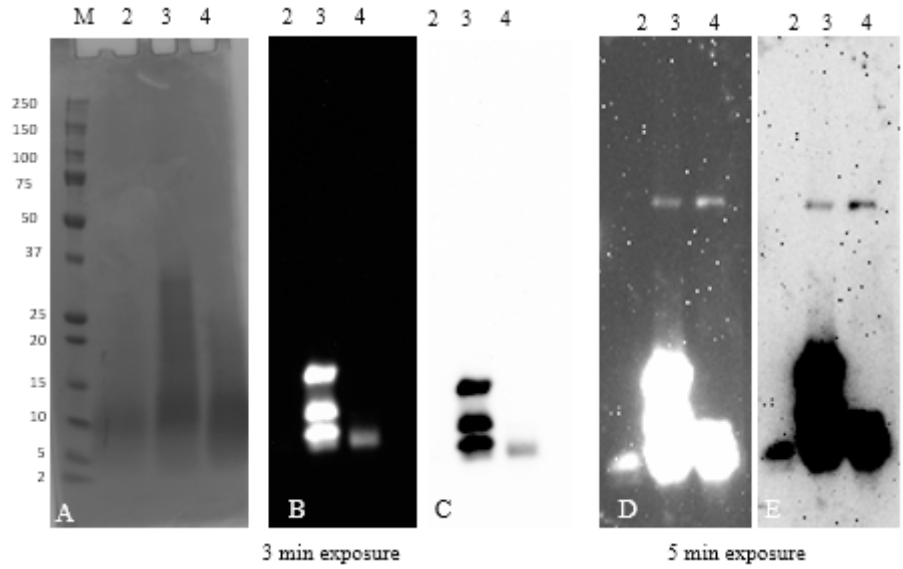

Figure 6

P. pratense-pollen western blotting results for serum 27121 (Table 1). Pollen protein extracts in 1X PBS were loaded (4 µg /15µl) to each well, SDS PAGE was run, and proteins were transferred to nitrocellulose membrane at a constant voltage of 30 volts and run for 90 minutes. A shows protein transfer to NC, B shows allergens on NC visualised under UVP Imager at 3 minutes exposure while C shows inverted image of B. D shows the same western blot visualized under 5 minutes exposure while E is its inverted image of D. M is precision plus protein marker. Lanes-2, 3 and 4 are R1, R2 and R3 pollens proteins loaded at 25 °C non-reducing conditions.