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ORIGINAL PAPER

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

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Abstract

Phleum pratense is an allergenic grass that pollinates in spring in Pakistan. 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*-pollen and soils in the spring season 2022. Samples were collected from three climatic regions in Pakistan (R1, R2 and R3) with differences in mean monthly air temperature, mean monthly precipitation and elevation. The morphology of pollen was observed by light microscopy, scanning electron microscopy (SEM), biochemical fingerprint analysis, and composition of pollen were investigated by fourier-transform infrared spectroscopy (FTIR). The pollen-associated bacterial populations were identified through a Biolog GEN III microplate system. The pollen water-soluble proteins were isolated and stabilized in phosphate buffer saline (PBS) and tested for allergenicity responses through dot blots and western blots analysis. The morphology study found difference in pollen biochemical composition. Biolog identified *Brevibacterium epidermidis* and *Pantoea agglomerans* from *P. pratense* pollen. Protein extract quantification and sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) gel found decreased protein expression in R1 region pollen compared to R2 and R3 region pollen. Allergenicity studies found differential expression of beta-expansin and profilin allergens in pollen obtained from the three regions. Beta-expansin and profilin were suppressed in R1 pollen and expressed in compared to R2 and R3 pollen. This is the first study to identify *B. epidermidis* and *P. agglomerans* growth on *P. pratense* pollen. Variable allergen expression in *P. pratense* pollen has also been observed in different regions. Soil pH, an increase in mean monthly temperature and a decrease in mean monthly precipitation correlated with pollen biochemical composition, and reduced beta-expansin and profilin expression involved in pollen growth and development. The findings of this research are unique, which enhances basic knowledge and understanding of *P. pratense*-pollen associated microbiota and climate change impacts on the pollen allergen expression.

Keywords Climate change · *P. pratense* · Biolog · *Brevibacterium epidermidis* · Allergen · Pollen

Introduction

Phleum pratense (Timothy grass), a member of the Poaceae family, which causes 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% of grass pollen sensitization in the United States of America and Europe (García-Mozo 2017). A single grass pollen contains 0.040 ng allergen that activates human immune system (Jung et al. 2021). Water-soluble proteins of the pollen encounter the mucosal layer of the nasal cavity

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during inhalation, and induce the 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 have been reported from 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 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* pollen may contain microbiota that could 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 pollenallergen 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. Soil pH and air quality affect pollen size (Lau and Stephenson 1993; Verscheure et al. 2023). Soil pH conditions affect *Ambrosia artemisifolia L.* growth and pollen IgE binding signal strength (Gentili et al. 2018). 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* produces 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 impact climate change has on *P. pratense*-pollen allergen expression and their biochemical composition and sensitization has not been 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 the effect of mean monthly temperature, mean monthly precipitation, soil pH conditions and elevation from sea level may affect pollen morphological features and allergen expression.

Materials and methods

Pollen sampling

P. pratense pollen and earth surface soils of respective locations were sampled in sterile zip lock bags in March 2022 from three distinct geographic regions of Pakistan. 5 g of the soil sample was suspended in 10 ml of deionized water from each region to determine the soil pH through Dynamica pHMaster pH meter serial #14,221,009 (McLean 1982). Regional altitudes were measured through a digital altimeter.

Region 1 (district Peshawar), region 2 (district Islamabad) and region 3 (district Kotli) are located at 331 m, 507 m and 609 m elevation from sea level, respectively. According to the Koppen-Geiger Climate Classification, Region 1 has BSh (dry, semi-arid and hot), whilst Regions 2 and 3 have Cwa (temperate, dry winter and hot summer) weather conditions (Adnan et al. 2017) (S1. Fig, https://ndmc.pmd.gov.pk/new/annual-climate.php?p=climate-classification). The mean monthly temperature during March 2022 for Region 1, Region 2 and Region 3 was 22.9 °C, 21.9 °C and 22.1 °C, respectively, and mean monthly precipitation during the period was 5.2 mm for R1, 59.5 mm for R2, and 21.1 mm for R3 (https://cdpc.pmd.gov.pk/Pakistan_Monthly_Climate_Summary_March_2022.pdf). Mean annual temperature and precipitation data from the past decade for the three regions was retrieved from Pakistan Meteorological Department and analysed for the past decade climatic trend. Two-way-ANOVA was applied on the mean annual temperature and precipitation dataset (2010–2021) of R1, R2 and R3 (S1. Table), and Tukey's multiple comparison test was applied to the means of pairs. For each region, mature pollen were collected from 3 to 5 nearby-located *P. pratense* plants on a sunny day by clipping the inflorescence of the

plant in sterile 50 ml falcon tubes. Only mature flowers of the plant were sampled, on a bright sunny day, to get fully developed pollen (about to spread in air). Samples were air dried at room temperature at a relative humidity of 30 g. m^{-3} for one week, and then tipped on a 500-µm pore size mesh. Isolated pollen 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 processed immediately or stored at room temperature in 1.5 ml sterile tubes in a wooden dark cabinet until further use.

Microscopy

Pollen grains (10 mg) were soaked in iso-propyl-alcohol (200 µl) and aqueous ponseau S stain for ten minutes to rehydrate. Folding effect was reduced by addition of aqueous ponseau S stain that rehydrated dry pollen and stained pollen red for microscopy. To minimize the folding effect, 30 pollen grains diameter readings were recorded for every region and the mean value for every region are presented respectively. 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. Pollen was directly kept on carbon patch-covered SEM stub holders. For each region 20 pollen 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). Two-way ANOVA was used to test variance in pollen diameter of the three regions, and Tukey's multiple comparison test was applied to the means of pairs (S2 Fig. and S1. Table).

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). Pollen samples were collected from the three regions, and each region sample was scanned three times. 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 for spectral peaks and principal component analysis (PCA) (Kenđel 2020), and one-way-ANOVA. Spectral datasets regions for protein (1500–1700 cm^{-1}) and lipid (2820–3000 cm^{-1}) were magnified from the entire FTIR spectra to make R1, R2 and R3 comparisons. The entire FTIR spectral region (500–4000 cm^{-1}) datasets were used for PCA and one-way ANOVA.

Identification of pollen associated bacteria

P. pratense pollen samples from the selected regions were shaken for 30 min 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 h at 33 °C. Morphologically similar colonies appeared on plates from all three samples of R1, R2 and R3 regions within 24 h. The bacteria were 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 bacteria's ability to utilize 71 carbon sources present in the microplate. Sub-cultured single colony from 24-hour bacterial culture on Biolog Universal Growth (BUG) media. A single colony from the BUG media petri plate through a sterile inoculator was taken and the inoculum swirled in inoculation fluid A (IF-A) to release the bacteria colony in the IF-A. IF-A turbidity was checked before and after the release of bacterial colony in the fluid, to ensure that the transmittance value of the IF-A was above 90%. After that, 100 µl IF-A was transferred to each well of the GEN III microplate through a multichannel pipette. Plates were incubated at 33 °C for 24 h, and plate readings recorded visually from the colour readings after 6 h and 22 h 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 bacteria from Biolog database.

Protein extraction

Total soluble proteins of *P. pratense* dry pollen 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 h, and filtered through grade 1 Whatman® filter paper 45 micrometre diameter. Defatted pollen was 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.

Two-way ANOVA was used to check variance in protein quantities.

Human sera selection

The allergenic human sera to *P. pratense* listed in Table 1 was purchased from Plasma Lab International (3128 Norton Avenue, Everett, WA 98,201 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 students at the University of Nebraska Lincoln USA (Ramadan et al. 2021). Ethical consents 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.

Allergenicity testing

The allergenicity of the pollen protein extract was confirmed by IgE binding tests 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) was 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 min. Manually set up image intensities to visualize and reduce the city background noise.

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 24,180-D	F /American	Dog and cat dander, *P. pratense*	Allergic rhinitis, asthma	135	10.5	1.53	0
PL 24,329-I	F /Caucasian	Multiple allergies	Allergic rhinitis	378	31.8	0	0
PL 25,218-G	M /American	Grass pollen	Allergic rhinitis	413	48.4	0	0
PL 29,148	M /Caucasian	NA	Allergic rhinitis and asthma and FEIA	525	0	0	0
19,362-CM	F /Caucasian	*A. hypogea*, *orchard grass*, *P. pratense*, *birch*, *ragweed*, *mugwort*	NA	377	61.6	20	31.2
23,508-JK	M /Caucasian	NA	NA	300	10.4	3.42	0
PL 27,121	M /Caucasian	NA	NA	4054	0	0	61
22,329 JE	M /American	NA	NA	*1953*	100	13.1	34
19,392 CS	NA/NA	*Soy*, *A. hypogea*		NA	0	0	0
9735 RE	NA/NA	NA	Anaphylaxis to peanut.	NA	0	0	0
22,104 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

Results

Analysis of mean annual temperature and precipitation data from years 2010–2021 by two-way-ANOVA at P value < 0.0001 showed significant differences in the three regions. Mean annual temperature was significantly different among the three regions. However, mean annual precipitation was significantly different between R1 and R2 and R1 and R3 regions. There was no significant difference between R2 and R3 (Fig. 1).

Pollen microscopy

SEM and light microscopy at 100 X found round shape of *P. pratense* pollen. Pollen exine layer has a scar like structure on each pollen that attached it to the pollen tube. The mean pollen diameter was 36.3 µm, 39.6 and 38 µm for R1, R2 and R3. Two-way ANOVA showed that R1, R2 and R3 pollen diameter means were significantly different at $P = 0.05$ (S2. Fig). R1, R2 and R3 soil pH was 7.3, 6.4 and 6.9 respectively.

Pollen fourier transform infrared spectroscopy (FTIR)

The pollen FTIR analysis of R1, R2 and R3 regions was done using the entire FTIR spectra (wavelength of 500–4000 cm^{-1}). In the entire FTIR spectra, proteins region (spectral wave length 1700–1500 cm^{-1}) and lipids region (spectral wave length 3000–2820 cm^{-1}) showed variation in proteins and lipids functional group peaks (Fig. 2I and II). The range of peak transmittance values for proteins were 12–19 (Fig. 2-I) and for lipids 10–25 (Fig. 2 II) in the respective spectra which differed for pollen from all three regions. The transmittance values were high for R1 region having elevated mean annual temperature and low mean annual precipitation in comparison 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. FTIR PCA of the three regions datasets showed differences in the three datasets. PCA score of the three datasets is more than 0.3 on the score plot which marks the difference in the three datasets (Fig. 2 III). The PCA value of R1 FTIR differs from R2 and R3 by 0.6 score which marks the dataset is different from R2 and R3 FTIR datasets. PCA analysis of entire FTIR datasets for

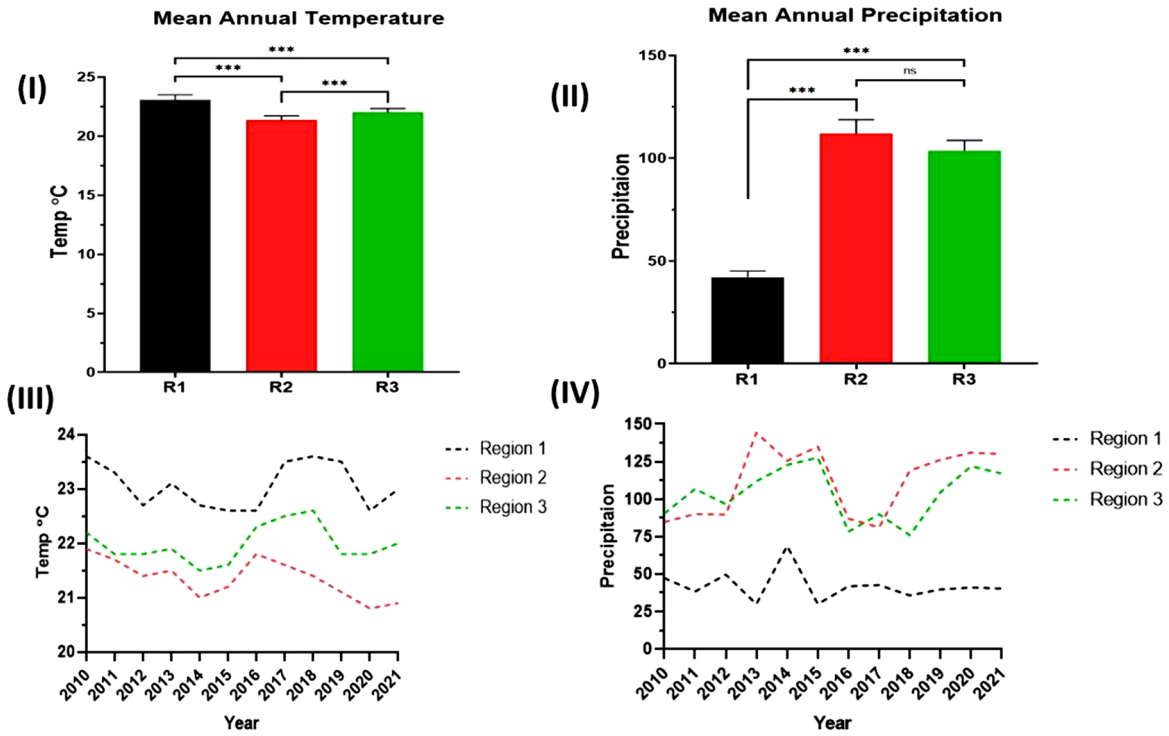

Fig. 1 Shows two-way-ANOVA and line graph of regions R1, R2, and R3 mean annual temperature (°C), and mean annual precipitation (mm) from year 2010 to 2021. (I) display two-way-ANOVA for mean annual temperature. Y-axis shows temperature, X-axis shows regions, and the star symbols on the top of the image show level of significance. (II) display two-way-ANOVA for mean annual precipitation. Y-axis shows precipitation, X-axis shows regions and the star symbols on the top of the image show level of significance. (III) shows trend of mean annual temperature and IV) shows trend of mean annual precipitation

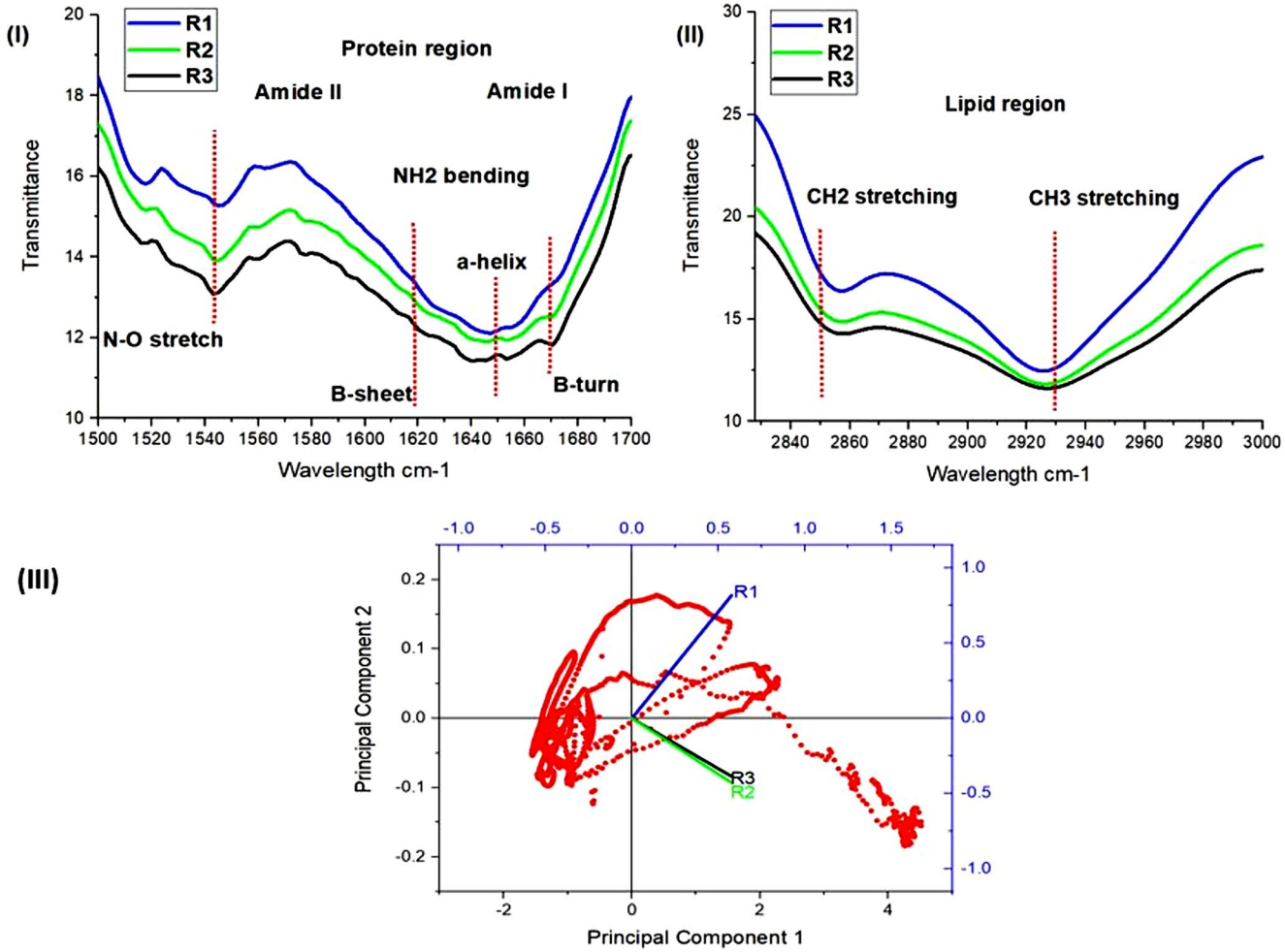

Fig. 2 FTIR analysis of *P. pratense* pollen collected from three regions R1, R2 and R3.(I) Shows *P. pratense* pollen FTIR spectral peak of protein region. (II) shows spectral peak of lipid region. In Fig. I and II, the Y-axis denotes transmittance values, and the X-axis shows wavelength. Blue, green and black 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. (III) shows PCA of the FTIR data sets. The red dots show data points while the three lines R1, R2 and R3 show vector forms of the datasets. Principal component I and principal component 2 are transmittance values of R1, R2 and R3 regions FTIR datasets shown in vector form. The value from 0.0–1.0 shows PCA score

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

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

all functional groups' regions (500–4000 cm^{-1}) is shown in Fig. 2 III which marked differences in the three datasets. Pollen FTIR one-way-ANOVA of entire FTIR datasets for all functional groups' regions (500–4000 cm^{-1}) showed that the population means are significantly different at $P \geq 0.05$ (S3. Fig).

Pollen associated bacteria Biolog identification

Biolog identification system using GEN III microplate identified *B. epidermidis and P. agglomerans*. The plate has 71 carbon sources, negative and positive controls. The bacteria 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 or partially 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 bacteria utilized the 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 and P. agglomerans.*

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 in 20 µl volume were (2.3 µg and 3.6 µg), (8.0 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. Two-way ANOVA demonstrated that pollen protein extracts from the three regions were significantly different ($P = 0.05$).

Dot blot analysis

Pollen protein extracts serum IgE binding results through dot blot showed allergenicity towards 16 human sera (see Fig. 3). Sera having strong IgE binding showed intense reaction and large diameter dots at 3 min 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. Strong IgE reactive sera showed IgE binding to 1:10 protein dilutions. Pure protein extracts and diluted protein extracts IgE binding is given in Fig. 3.

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.

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 min. 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

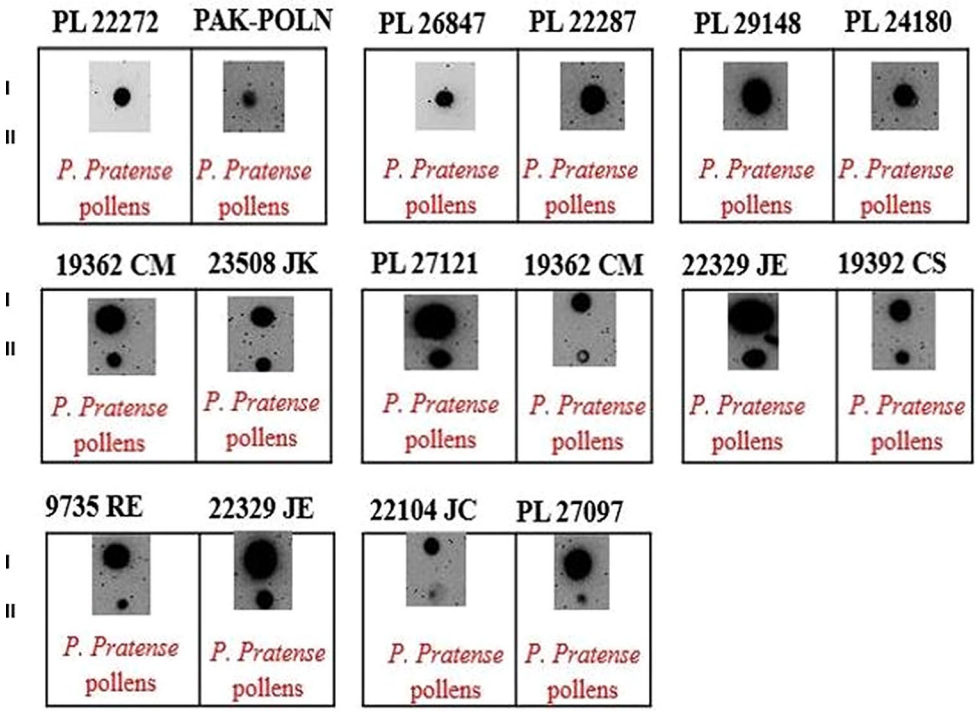

Fig. 3 *P. pratense* pollen-protein dot blot analysis. The dot in the box shows pollen allergen reactivity with primary antibody present in the serum of study participants. The bigger dots show pure pollen protein IgE binding, while the smaller dot denotes 1:10 1X PBS diluted protein IgE binding. The number above the box represents the serum identification number used in the study. Pure and 1:10 diluted pollen proteins IgE binding is shown by roman numbers I and II respectively on the left side of the image

bands under UVP imager. PL 22,287 serum showed about 32 kDa bands putative Phl p 1 allergen for R2 and R3 (Fig. 4I). For Pak Poln serum and 26,847, proteins transferred to NC membrane showed putative allergen, either Phl p 2 or Phl p 3 for R2 and R3 (Fig. 4II, and Fig. 4III). 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. 4IV).

Proteins transferred to NC membrane showed bands for R2 and R3. 27,121 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 min exposure under UVP imager. The same NC membrane showed about 55 kDa allergen Phl p 4 for R2 and R3 samples (Fig. 5).

Discussion

Pollen proteins can easily come across the nasal epithelial cavity during breathing, where pollen allergens cause allergic reactions. Pollen based allergies are increasing with climate change due to pollen season extension, increases in the amount of pollen and changes in allergenic protein concentrations (Ziska 2021). Climatic conditions have also a pronounced effect on pollen morphological and biochemical features, and protein expression profiling. Air quality, soil and water conditions affect pollen size (Lau and Stephenson

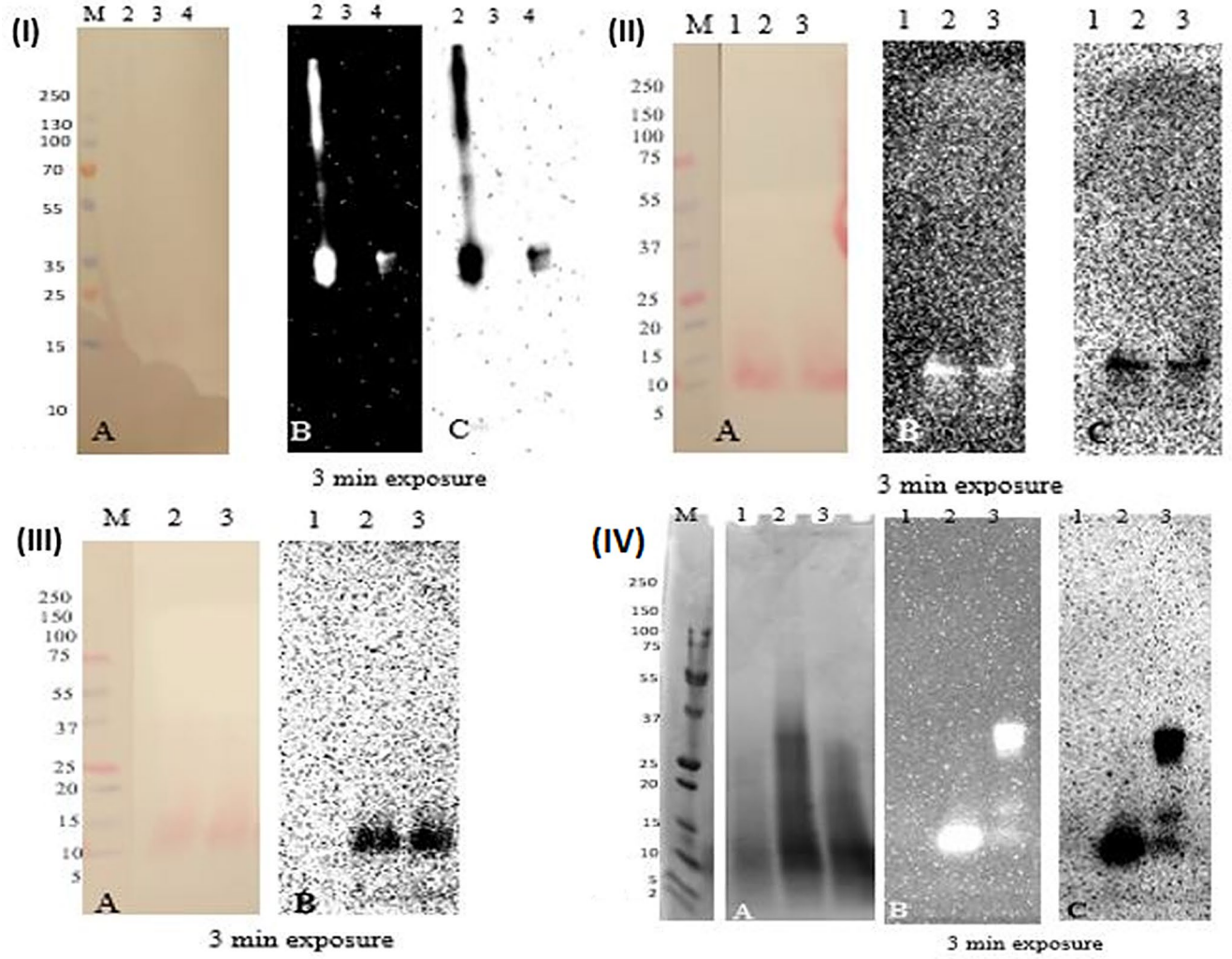

Fig. 4 *P. pratense*-pollen western blotting results for serum samples PL 22,287, Pak Poln, 26,847 and 9735 RE respectively (Table 1). In the fig I, II, III and IV, A shows protein transfer to NC, B shows allergens on NC visualised under UVP Imager at 3 min exposure while C shows inverted image of B. M is precision plus protein marker. Lanes 1, 2 and 3 are R1, R2 and R3 pollen proteins loaded at 25 °C non-reducing conditions

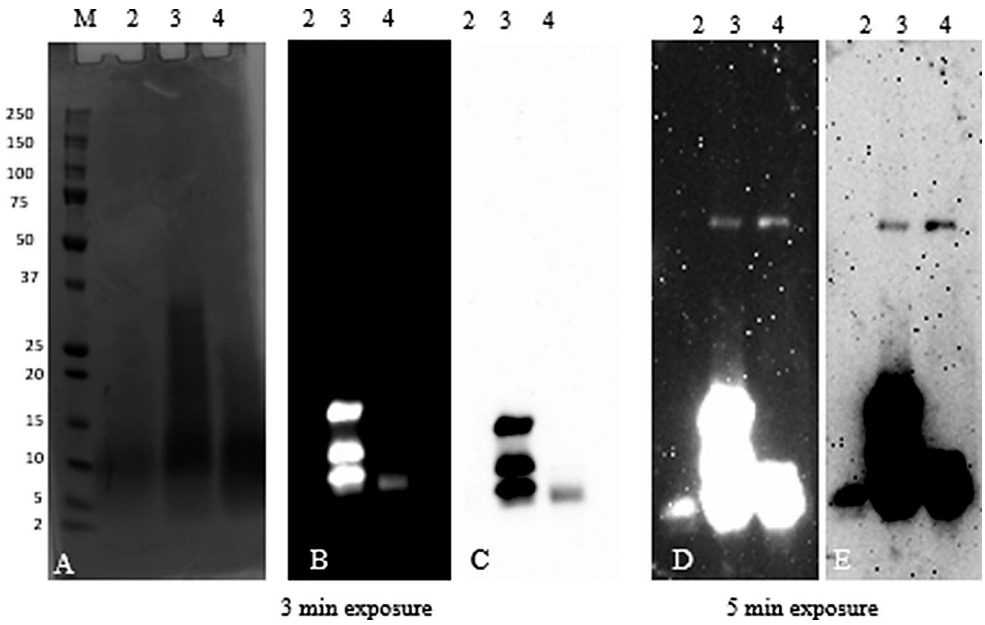

Fig. 5 *P. pratense*-pollen western blotting results for serum 27,121 (Table 1). Pollen protein extracts in 1X PBS were loaded (4 µg /15µl) to each well, SDS PAGE was run, and proteins were transferred to nitrocellulose membrane at a constant voltage of 30 volts and run for 90 min. A shows protein transfer to NC, B shows allergens on NC visualised under UVP Imager at 3 min exposure while C shows inverted image of B. D shows the same western blot visualized under 5 min 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 pollen proteins loaded at 25 °C non-reducing conditions

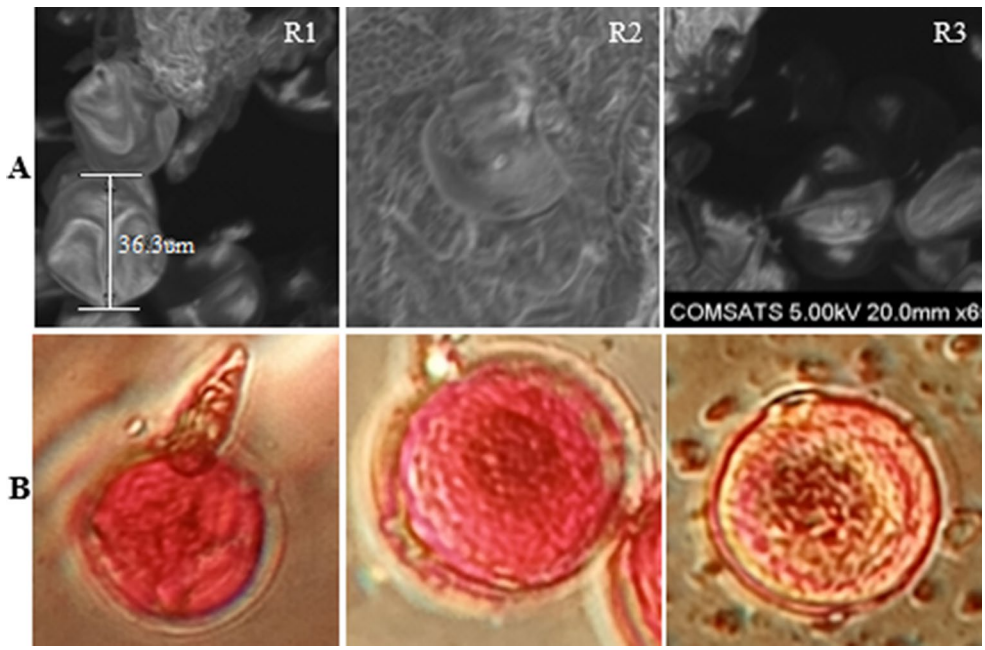

Fig. 6 Light microscope analysis of *P. pratense* pollen at 100X resolution from three climatic regions R1, R2 and R3 (**A**) Scanning electron microscopy (SEM) analysis, the black line in the centre shows the average diameter of pollen calculated through SEM in-built microscale. (**B**) light microscopy images of pollen of R1, R2 and R3 regions coloured with ponseau S stain and captured through light microscope having circular and dotted morphology

1993; Verscheure et al. 2023). Environmental conditions have been found variable on different species pollen. *P. pratense* is an abundant perennial grass that produces allergenic pollen. In this study, we have found that significant differences are present in the environmental conditions of the three regions (see Fig. 1). These regions elevation from sea level, soil pH, mean annual temperature and mean annual precipitation vary from each other. Our study findings are synonymous with the findings of Adnan et al. (2017), who established that these three regions occur in three distinct agro-climatic zones. They have divided the whole region of Pakistan into 8 distinct agro-climatic zones based on observational data of precipitation and evapotranspiration from 1951 to 2010. We have investigated the effect of mean monthly temperature, mean monthly precipitation, elevation from sea level, and soil pH under natural conditions in three different regions (R1, R2 and R3) on *P. pratense* pollen biochemical composition, and pollen allergen expression. The mean pollen diameter of the R1 region (36.3 µm) was smaller than R2 (39.6 µm) and R3 (38 µm) (see Fig. 6). Pollen diameters were significantly different in all regions ($P = 0.05$). R1 region has increased mean monthly temperature and decreased mean monthly precipitation in comparison to R2 and R3 regions. In previous investigations (Jung et al. 2021), it was observed that decreases in mean pollen diameter (lower size & weight) of *P. 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 pollen to a species (Radaeski et al. 2016). Soil pH of R1, R2 and R3 was 7.3, 6.4 and 6.9 respectively. The soil pH may have affected pollen allergen expression. In a previous study conducted on *Ambrosia artemisifolia L.*, soil pH growth was associated with pollen IgE binding signal. The plant pollen protein developed at pH 5 soil has higher IgE binding signal in comparison to pollen developed at pH6 and pH7 (Gentili et al. 2018).

Water soluble protein content was lower in R1 region pollen in comparison to R2 and R3. These findings are similar to those of Jung et al. (2021), where they showed that *P. pratense* pollen has lower protein and allergen content under drought stress conditions. 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 (Jung et al. 2021). Under drought stress conditions, *P. pratense* pollen showed lower size and weight under drought stress condition. Our results showed that R1 (dry semi-arid conditions, S1. Fig) 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.

FTIR analysis showed differences in pollen biochemical profile as the spectral peaks differ in pollen of all three regions (see Fig. 2-I-II). Region R1 has elevated mean monthly temperature and low mean monthly precipitation (22.9 °C, 5.2 mm) but lower elevation from sea level in comparison to R2 (21.9 °C, 59.5 mm) and R3 (22.1 °C, 21.1 mm). Region R1 has elevated mean monthly temperature and low mean monthly precipitation (22.9 °C, 5.2 mm) but lower elevation from sea level in comparison to R2 (21.9 °C, 59.5 mm) and R3 (22.1 °C, 21.1 mm). Therefore, FTIR spectral peaks reflected that R1 pollen 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 pollen of these two regions showed similar spectral peaks. Spectral differences in pollen growing in different environmental conditions correlate to differences in pollen protein, carbohydrate, lipids and sporopollenin (Zimmermann and Kohler 2014). They studied 300 different plant species pollen 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. Similarly, one-way ANOVA for R1, R2 and R3 FTIR datasets of entire spectral regions showed that population means are significantly different at $P \geq 0.05$ (S3. Fig). 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 and gram negative, oxidase negative and catalase positive bacteria from *P. pratense* pollen, having optimal growth at 33 °C. The bacteria formed grey white and yellow colonies on LB agar for 24 h of incubation period. Biolog Gen III plate testing identified the bacteria as *B. epidermidis and P. agglomerans*. Previously, *B. epidermidis* was discovered from cheese, milk and human skin isolates that formed 2–4 mm grey white colonies on nutrient agar media after 24 h incubation between 30 and 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). *P. agglomerans*, an opportunistic pathogen was identified from the flowers of *Epipactis helleborine* that strengthens the findings of our study (Jakubska-Busse et al. 2021). Comparisons of allergenic and non-allergenic plants pollen and pollen 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

pollen (see Fig. 7). 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. In the presence of adequate humidity, an increase in temperature induces vegetative and generative functions in Poaceae (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 mean monthly temperature, mean monthly precipitation, elevation from sea level and soil pH conditions in the three regions have caused differences in the PBS soluble protein expression. Water stress, heat stress and air pollution induce 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 Research square preprint). 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

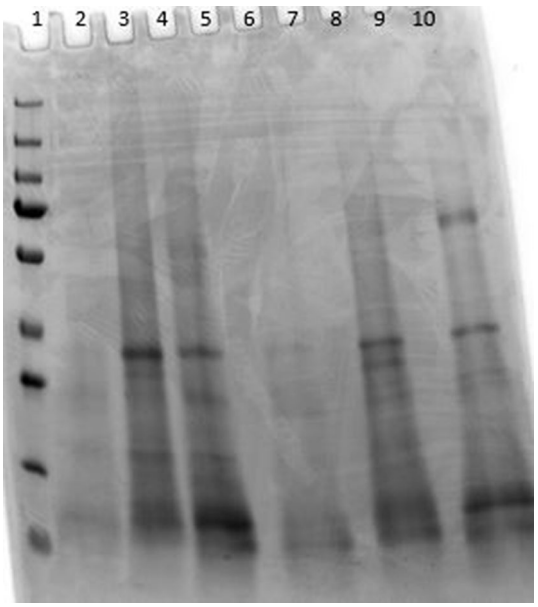

Fig. 7 Invitrogen 10–20% tris glycine premade gel run for 90 min of *P. pratense* pollen protein under 130-volt constant voltage. *P. pratense* defatted pollen protein (4 µg /15µl) extracted in 1X PBS were loaded in each well. Lane 1 is precision plus protein marker, lane 2 is *P. pratense* Peshawar 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

stress (Mascarenhas and Crone 1996). The decrease in *P. pratense*-pollen weight, and protein content variation under drought and heat conditions has been reported by Jung et al. (2021). The authors found significantly increased allergen content in control treatments as compared to heat treatments (warming treatment, warming + drought treatment) (Jung et al. 2021). Our study finds that putative allergens' expression in *P. pratense* pollen has also been changed in response to 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, but its expression was missing in R1. Different environmental conditions of R1 affected Phl p 1 allergen compared to R2 and R3 (see Fig. 4I). Similarly, R2 and R3 have different mean monthly precipitation and elevation from R1.

Lol p 1 (27 kDa beta-expansin) is a major *Lolium perenne* allergen, and a member of the 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. 4-II, Fig. 4-III and Fig. 5 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 min exposure in UVP imager 8.5 while western blot 5 min 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 causes pollen infertility (Pacini and Dolferus 2019; Santiago and Sharkey 2019). Proline amino acid levels decrease under heat stress conditions (Santiago and Sharkey 2019) which strengthens our findings where elevated temperature at R1 has reduced allergen expression. Similarly, the western blot 5 min exposure in UVP imager 8.5 showed very faint expression of Phl p 4 in R2 and R3

samples (see Fig. 5). 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. 5). 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. Increase in temperature and decrease in precipitation inhibit the putative allergen expression in R1 region pollen. Differences in environmental conditions between the three regions affect the expression of Phl p 12.

Putative allergens Phl p 2 or Phl p 6 are expressed in R2 and R3 only (see Fig. 4-IV). Additionally, Phl p 12 and Phl p 5 showed expression in R3 only. 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 study's findings elaborate how variations in climatic conditions influence *P. pratense*pollen size, biochemical composition and allergen expression. R1 region *P. pratense*-pollen were smaller in comparison to R2 and R3 region, and their respective FTIR spectral peaks marked differences in pollen biochemical composition. SDS PAGE showed loss of certain protein bands for the R1 sample in comparison to R2 and R3 samples. 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. Additionally, we report for the first time *B. epidermidis and P. agglomerans* from *P. pratense* pollen through Biolog identification system that may have a role in *P. pratense*-pollen allergies which need to be further investigated. These findings suggest that soil pH conditions, elevation from sea level, increase in temperature up to 1 °C and decrease in precipitation may affect pollen biochemical composition and allergens expression during pollen development.

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Author contribution 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.

Declarations

Data availability The data that support this study are available from the corresponding author upon appropriate request.

Conflict of interest The authors declare no conflict of interest.

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