Metabolomic characterization of plant exudates and their correlation with plant defense systems

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Chapter 5

Constitutive plant latex: A metabolic constrained system against infection and infestation

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Chapter 5

ABSTRACT

Plant latexes are thought to be the first barrier against natural enemies and are thus an excellent model to study interactions between plants and other organisms. However, their exact roles are still generally unknown. A closer knowledge of latex metabolic variation could provide an insight of the defensive chemical selection of plants. This idea is based on the hypothesis that the variation of the latexes' metabolomes is a response to selective pressure and should thus be affected differently from other organs. To prove this, a multi-analytical platform-based metabolic profiling was used to study three *Euphorbia* species collected in various regions. Regardless of environmental factors, the chemical profiles of latexes were much more conserved than that of leaves and roots. In all the species, the level of polyisoprenes and terpenes in latexes was found to be extremely higher than in other tissues. These metabolites commonly found in latexes were suspected to be involved in a systemic defense. In the first place, polyisoprenes were involved in physically retarding the movement of pathogens and herbivores. As a secondary chemical barrier, terpenes, especially, 24-methylenecycloartanol was shown to fight pathogenic fungi. These results, together with the known roles of the enzymes (e.g. proteolysis), prove that latexes are part of a cooperative defense system that included biochemical, biological, chemical and physical resources.

Keywords: Latex; metabolic variation; chemical selection; 24-methylenecycloartanol; mechanical barrier; constitutive defense.

Chapter 5

1. Introduction

All living organisms are constantly challenged by diverse environmental factors including biotic and abiotic stress[1]. In order to survive as a species, during their interactions they must develop responses, evolving genetically and/or phenotypically (Andrew et al. 2010). Thus, the biological and chemical complexity of life can be considered to be, to some degree, the consequence of the genetic diversity modulated by ecological interactions. Especially in plants, sessility has acted as a strong evolutionary pressure, resulting in the development of diverse physical and biochemical tools as sophisticated adaptive systems. This has allowed them to persist throughout the time in diverse types of ecosystems (Bucharova et al. 2016). Among these tools, plant exudates have attracted great interest because of their adaptive origin, having resulted from their coevolution with other organisms including insect herbivores and microorganisms (Konno 2011). Plant exudates represent one of the surface defense layers associated with both primary and secondary defense systems. Among plant exudates, latexes are the most interesting, not only due to their commercial value, but also because of their distinctive chemistry which is assumed to have an ecological potential as a primary barrier in response to exogenous factors (Agrawal and Konno 2009).

Though the specific role of individual latex metabolites are still unknown, as a whole, their metabolomes are highly distinctive, exhibiting chemical fingerprints that differ both quantitatively and qualitatively from those of other organs like leaves or roots (Seiber et al. 1982; Konno et al. 2004; Konno et al. 2006). Furthermore, the bioactive metabolite contents in latex before and upon herbivory or pathogenic attacks at damaged points can vary, as their concentration is locally increased (Ball et al. 1997; Hölscher et al. 2016; Gorpenchenko et al. 2019). Therefore, understanding the role/s of latex metabolites and their part in the complex mechanisms behind plant defense systems could contribute to the greater picture of chemical selection in the evolution of plant defense. Together with the chemical selection, the degree of the metabolic variation in defensive exudates such as latexes could also determine the success of this type of defensive system. Thus, several fundamental questions arise: are the latexes from different species chemically

similar? To what degree do environmental factors affect the chemical variation of the latexes? Do specialized metabolites exert their biological activity individually or do they act in a complementary or even synergistic manner with other latex components? If so, how do they interact? By answering these questions, the detailed roles and mechanisms behind latex chemistry and its variation could be understood.

To answer these questions, an experimental design using a holistic approach was adopted. For this, a model system consisting of a plant sample set with different genetic (species) and environmental (geographical origin) backgrounds was chosen. The sample set included wild *Euphorbia* specimens of *Euphorbia palustris*, *Euphorbia amigdaloides* and *Euphorbia glareosa* collected in different locations in Serbia. The metabolic composition of latexes, leaves, and roots were studied with ^{1}H NMR and LC-MS-based metabolomics methods, while GC-MS and HPTLC-DART-MS were used as supplementary tools for the analysis of targeted metabolite groups. Based on the metabolomics results, the anti-herbivory, antibacterial, and antifungal activities of the latexes were assayed and the degree of chemical and biological variation was correlated in order to deduce the role of individual metabolites. This holistic approach revealed the existence of an efficient defense system in latexes.

2. Results and discussion

Plants are thought to have developed their own defense systems differently to other organisms. However, the detailed mechanisms behind these systems are largely unknown. Plant latexes have been considered for a long time to be highly valuable substances. This interest arises mainly from their extremely high concentration in selected chemicals such as natural rubber (1,4-*cis*-polyisoprene) with important industrial applications, and from their potential use as a high hydrocarbon sink for fuel and chemical feedstocks uses (Kalita 2008). However, further studies have revealed the presence of bioactive metabolites and proteins with insecticidal, deterrent, antibacterial, antifungal, and cytotoxic activity, pointing to a potentially important role in the mechanisms behind plant defense. Moreover, latexes

have also recently been recognized as a good ecological model for the understanding of plant-herbivores interactions (Konno 2011).

The chemical composition of latex includes three major fractions: polyisoprene polymers (rubber), proteins and small specialized metabolites. Among these, proteins have been relatively more studied due to their obvious biological functions against pathogens and herbivores. In the case of rubber, the research has been limited to the coagulation process necessary for industrial purposes, but little is known of their role in defense mechanisms. The chemical and biological diversity of latexes is provided by their specialized metabolite content, similarly to other tissues. However, despite their proven biological activities, the potential roles of these specialized metabolites *per se* or eventually in cooperative roles with other components of latexes such as rubbers or proteins related to the plant response to environment have not been studied. Thus, some fundamental questions still remain unanswered: are the latexes from different species similar? How do environmental factors affect the degree of chemical variation of the latexes? Do specialized metabolites display their biological activity individually or complementarily, or even synergistically, with other latex components?

In this regard, due to their anatomical location, latexes might be one of the first mechanical and chemical barriers of plants against herbivores and microorganisms. Were this true, latexes should possess distinctive metabolomes from plant tissues. Moreover, to be able to fulfill their basic role as a first barrier, the latex metabolome should be composed of a few selected metabolites at high concentrations, exhibiting a semi-conserved constitutive chemical composition which is relatively unaffected by environmental factors. Thus, a lower variation in latexes could imply that they are involved in basic functions, e.g. as a first barrier against general predators or pathogens. Conversely, if the variation was greater, it could indicate that latexes might be related to specialized roles such as a reaction to subtle environmental changes including the attack of location-specific predators or pathogens. In order to investigate and compare the effect such factors on the metabolome of latex with that observed in other tissues (leaves and roots), three *Euphorbia* species were collected from nine locations in

Serbia. The idea was to gauge the similarity in the metabolic variations of latexes and their bearing tissues.

To compare the metabolic variation of each tissue, their ^{1}H NMR spectral data was subjected to multivariate data analysis (MVDA). Firstly, the spectra of each tissue from the three locations were analyzed by principal component analysis (PCA). As shown in supplementary Fig. 1, each *Euphorbia* species exhibited a distinctive metabolome in all the profiled tissues. This was not unexpected as the species effects are known to be a significantly influential factor. However, interestingly when comparing the metabolic variation related to geographical origin of each tissue, latexes were found to show much less variation than leaves and roots. This was also confirmed by further MVDA, a soft independent modelling of class analogy (SIMCA) analysis, in which a PCA model is built for each class and the distance (DModX) between models is measured to determine their similarity or dissimilarity (Eriksson et al. 2011). For all the species, the values of DModX were calculated for latexes, leaves and roots and the obtained values were logarithmic transformed and averaged to show the degree of variation. In this model, the higher the DModX, the higher the metabolic variation. As shown in Fig. 1, the SIMCA model confirmed that the variation in metabolomes among latexes was much lower than between leaves and roots in all the tested species.

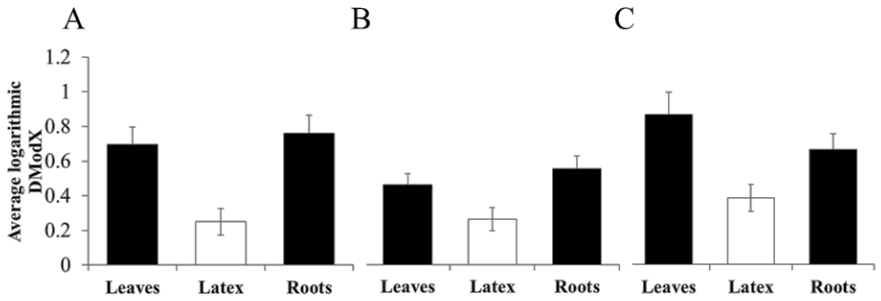

Fig. 1. Logarithmic averaged distance to the model (DModX) values in latex, leaves and roots of three Euphorbia species. (A) *Euphorbia amygdaloides.* (B) *Euphorbia glareosa*. (C) *Euphorbia palustris*. The averaged values represent the mean (n = 30 ± standard error) of the DModX from a soft

independent model of class analogy (SIMCA) analysis of all samples of each tissue per species.

To confirm this, the Q^2 value (an indicator of correlation) of partial least square-discriminant analysis (PLS-DA) was calculated. The Q^2 value reflects the degree of correlation between the chemical data set and the classes (geographical origins) (Eriksson et al. 2011) and can thus be regarded as a measurement of their degree of total correlation. Thus a smaller correlation degree would be expected between the latex chemical set and their geographical origins. As shown both by PCA and SIMCA results, geographical factors have highly differential effects on the metabolic variation of the leaves (Q^2 = 0.96 ± 0.002) and roots (Q^2 = 0.91 ± 0.012) while latexes showed much less correlation (Q^2 = 0.53 ± 0.220) indicating that they are not affected so much by their geographical origin.

All the data analyses confirmed that the metabolome of latexes is more conserved than those of leaves and roots, and presumably less influenced by environmental factors. It is generally believed that plant metabolomes are largely influenced by environmental factors. The difference in chemical diversity in ecotypes has been well documented for a few species and interpreted as an adaptive response to specific biotic and abiotic factors found in different ecosystems (Shelton et al. 2004). Each plant species evolves differentiated chemical responses according to their survival requirements both at a population and individual levels (Shelton et al. 2004). However, contrary to this general rule, in the case of latexes their metabolome varies much less than other tissues. From a plant defense perspective, this lower metabolic variation of latexes suggests that they might play a primary and general role in defense, anticipating the more complex inducible response. Were this the case, it could also be supposed that latexes could be less influenced by the species factor than by geographical locations. Among the tested species the metabolic variation in latexes (DModX = 0.76 ± 0.07) was lower than in roots (DModX = 1.05 ± 0.08) but showed no relevant differences with that of leaves (DModX = 0.76 ± 0.06).

The next step consisted in the identification of the discriminating metabolites between latexes and other tissues. This was performed using their ^{1}H NMR spectra. The lower chemical variation observed in latexes indicated

that there are more common metabolites among latexes than in leaves and roots. The comparison of the ^{1}H NMR spectra of leaves, roots and latexes showed extremely higher levels of triterpenes in latexes than in the other tissues. Polyisoprenes in particular were present in high amounts in latexes as compared to leaves and roots in all the studied *Ephorbia* species. The latex-specific triterpenes were elucidated by typical methyl signals in the δ 0.70 – δ 1.90 range (Fig. 2). Additionally, the cyclopropane moiety in the structure of cycloartanol was confirmed by two doublets at δ 0.55 (d, J = 4.0 Hz) – δ 0.35 (d, J = 4.0 Hz) assigned to the 19-*endo* and 19-*exo* protons, respectively (Fig. 3). The cycloartanols were further confirmed by DART-MS and GC-MS analysis. The direct mass analysis showed 441.4167 *m/z* and 458.4429 *m/z*, which were assigned to the $[M+H]^+$ and $[M+NH_4]^+$ adducts (mass error < 7 ppm), respectively, from 24-methylenecycloartanol. In terms of relative quantitation performed using the integration of the H-19 resonance, it was found that the content of the cycloartanol-type triterpenes in latex was over eight times higher than in other tissues (Figure 3). Actually, the 24-methylenecycloartanol content accounted for almost 16 % of dry weight of latexes. The GC-MS analysis confirmed this, showing that a few other cycloartane analogues such as 24-methylenecycloartatnone and 24-methylenecycloartanol acetate were more abundant in latexes than in other tissues.

Triterpenoids have been identified in *Euphorbia* species because of their chemotaxonomic importance (Ponsinet and Ourisson 1968; Mahlberg and Pleszcynska 1983) and many of them have been suggested to be chemo-markers for certain taxons (Mahlberg et al. 1987; Mahlberg et al. 1988). Previous reports on latex chemical composition, mention rubber and triterpenes as the main components of rubbery latexes (Konno 2011; Agrawal and Konno 2009) assuming that given their proximity in biosynthetic pathways, their coexistence in laticifers might have occurred during evolution (Mahlberg 1985; Piazza et al. 1987a; Piazza et al. 1987b; Nemethy et al. 1983). This hypothesis does not explain the selection of a specific terpenoid in a concentration eight times higher than in other tissues as observed in our results. The value of triterpenes in latexes should be functionally coupled to that of rubber. In other words, the lower degree of chemical variation in

latexes strongly suggests their biological activities are limited only to a basic protection in an early stage of defense, rather than to a specialized role.

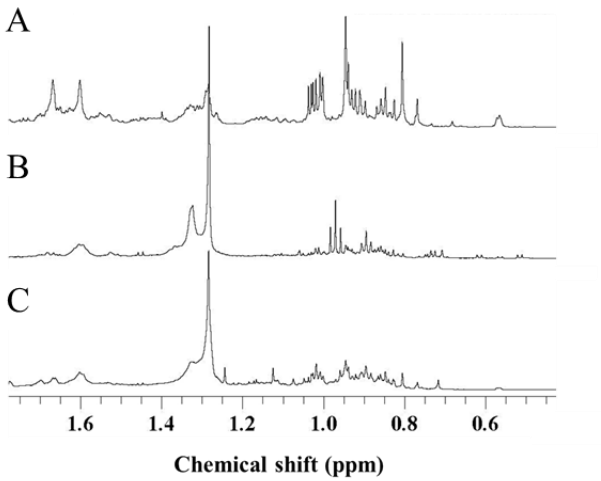

Fig. 2. Representative ^{1}H NMR spectra (600 MHz, MeOD + HMDSO) from latex, leaves, and roots. A, latex. B, leaves. C, roots. Range of δ 0.4 – δ 1.8 of latex reveals a higher concentration of terpenes resonances.

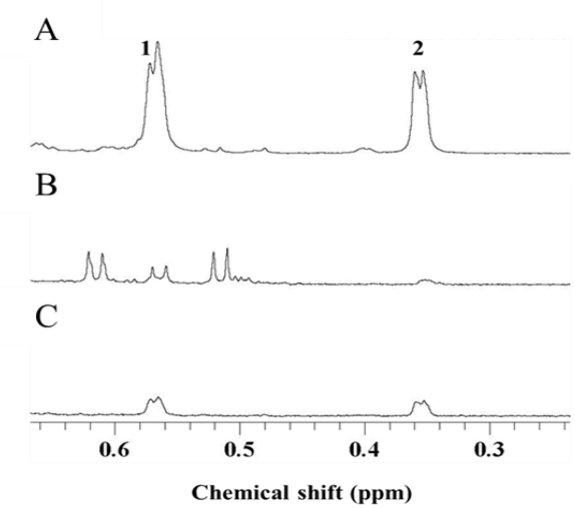

Fig. 3. Representative ^{1}H NMR spectra (600 MHz, MeOD + HMDSO) from latex, leaves, and roots. A, latex. B, leaves. C, roots. Two doublets at (1) δ 0.55 and (2) δ 0.35 correspond to the 19-*endo* and 19-*exo* protons, respectively, of the cyclopropane ring of 24-methylenecycloartanol with a concentration ~8 times higher in latexes than in the other two tissues.

The LC-MS analysis of the latex samples provided further details of their metabolic variation. Similarly to the ^{1}H NMR-based analysis, the variation among latex samples from different locations was found to be low, showing no separation by geographical origin in *E. palustris* and *E. glareosa*. However, some species-specific metabolites such as alkaloids were identified (Table 1). Alkaloids were found to be specific for *E. glareosa* and *E. amygdaloides* while acyl sugars were found to be more related to *E. palustris*. Latex is believed to play a primary role in the defense of plants against a broad range of natural enemies, acting as an immediate barrier until other induced defense mechanisms are active. Thus, if the variation in the chemical composition of latexes is lower than in other tissues, the variation in their anti-herbivore activity should consequently also be lower than that of the other tissues. To prove this hypothesis, latexes, leaf and root extracts were challenged with a generalist herbivore, *Mamestra brassicae*. In terms of anti-herbivory activity, the results showed significant anti-feeding effects of all three tissue extracts in two of the three tested species compared to the negative control diet ($p < 0.05$).

This could be the outcome of tissues with differentiated metabolomes but similar ecological functions such as constitutive defense. Nonetheless, even with a more limited number of metabolites than other tissues, latexes exhibited a very efficient metabolome designed as a defense against herbivores. In the case of *E. palustris* the latex showed a lower average than the negative control but not statically significant ($p = 0.12$), this value however denotes a trend pointing out to a positive anti-herbivory activity. For this specific case, the data of two samples, one from Borča and one from Čenta, were rather outliers for that group of samples resulting in a big standard error, which might cause the no significant differences with the negative control. However, when analyzing the variation of the bioactivity for each species provided by the standard error, the variation of the anti-herbivore activity in its latex was lower than that of leaves and roots at least in two of the three studied species (Fig. 4). This result suggested that the lower chemical variation of latexes was consistent with a more homogeneous variation in the biological activity against *M. brassicae*.

Table 1. Identified discriminant metabolites in latexes from *Euphorbia palustris*, *Euphorbia amygdaloides* and *Euforbia glareosa* collected at different locations of Serbia.

Compound	OM	ME (ppm)	IM	Species		
				Euphorbia Palustris	*Euphorbia amygdaloides*	*Euphorbia glareosa*
nicandrose E	650.3532	2.83	Neg	Ø	–	–
sibiromycin[a]	648.3371	0.08	Neg	Ø	–	–
Asperazine	664.2791	-1.06	Neg	–	Ø	–
milliamine C	706.2854	-5.14	Neg	–	Ø	–
Rankiniridine	552.2486	2.66	Neg	–	–	Ø

Compound	OM	ME (ppm)	IM	Geographical origin								
				Bo	Če	Ša	Mj	Av	Ko	Tb	Dp	Zb
solanoglycosydane I	576.4099	-6.80	Neg	Ns	Ns	Ns	Ø	–	–	Ns	Ns	Ns
Plactin	644.4008	-0.16	Neg	Ns	Ns	Ns	Ø	–	–	Ns	Ns	Ns
aphanamolide B	706.2830	-0.84	Neg	Ns	Ns	Ns	–	Ø	–	Ns	Ns	Ns
manadoperoxide J	406.7664	1.62	Neg	Ns	Ns	Ns	–	Ø	–	Ns	Ns	Ns
stelleralide C	666.2976	-9.55	Neg	Ns	Ns	Ns	–	–	Ø	Ns	Ns	Ns
C36H36N2O8	624.2411	-0.08	Neg	Ns	Ns	Ns	–	–	Ø	Ns	Ns	Ns
Pentaglycerol	388.1910	-8.76	Pos	Ns	Ns	Ns	Ø	–	–	Ns	Ns	Ns

Ø: presence of the compound, OM: observed mass, ME: mass error, IM: ionization mode, –: absence of the compound, Ns: no cluster separation by PCA, Neg: negative ionization mode, Pos: positive ionization mode. [a]Derivative: 9-O-(4,6-Dideoxy-3-C-methyl-4-(methylamino)-α-L-mannopyranoside),[b]possibly grossamide, heliotropamide, or lyciumamide B.

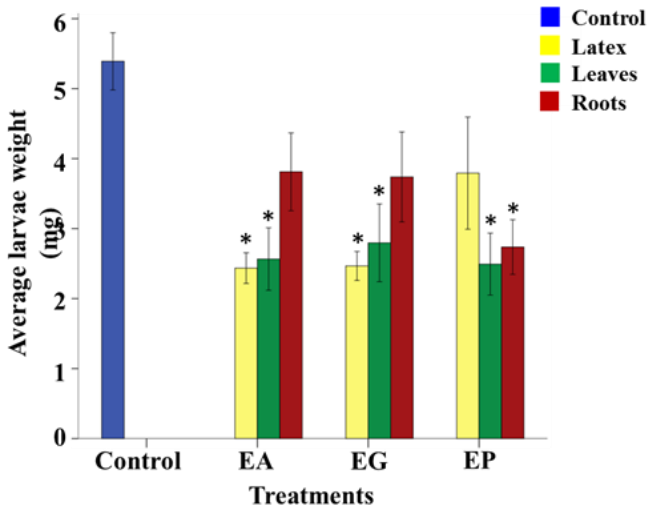

Fig. 4. Anti-herbivore activity from latex, leaves, and roots of *Euphorbia amygdaloides*, *Euphorbia glareosa*, and *Euphorbia palustris*. The values represent the mean value (n = 30) ± standard error. The * represent significant differences between the treatment and the control on a Least-square means test ($\alpha = 0.05$).

The biological activity of latexes against *M. brassicae* showed less variation among the latex samples irrespective of their geographical locations. Thus, the low variation and relatively simple metabolome, rather than a disadvantage proved to be able to provide a more stable and constant protection against herbivores than those of leaves and roots that are more susceptible to environmental variations as they adapt to them through adjustments in their metabolome. These results indicate a long co-evolution between the plant species and diverse interactions of plant-herbivores to develop a selective or limited number of compounds against a broad range of natural enemies (Fim and Jones 2003). In particular, this conserved metabolome could be more beneficial for a primary role in defense when dealing with generalist organisms, combining constitutive defense compounds and mechanical traits. In this regard, it is well-known that when high herbivore pressure conditions are present, like in latex bearing species, it was found that the constitutive defenses could create a cost-benefit balance in the plant fitness (Moore et al. 2014; Andrew et al. 2007).

In nature, the most efficient way to deal with a wide range of natural enemies and to avoid the fast development of resistance with a minimal use of resources is to employ a synergistic blend of traits (Richards et al. 2010; Richards et al. 2012). Were this true for latexes, a possible synergistic effect between their ingredients is to be expected. To test this potential cooperative mechanism, the activity of polyisoprene was firstly tested against bacteria.

When the latex extracts were tested on *Pseudomonas viridiflava*, *Pseudomonas fluorescens* and *Pseudomonas putida*, considered being general pathogens, they did not display any antibacterial activity. A possible explanation for these results was that given the high concentrations of polyisoprenes detected with the metabolic screening, latexes provide a physical or mechanical defense mechanism rather than or added to, some chemical toxicity. This assumption fits well with the hypothesis that latexes have a general and broad function of defense in the early stage of plant defense. To prove this hypothesis, a mimicking experiment was performed. A layer of poly-*cis*-1,4-isoprene was spread on agar plates inoculated with the bacteria and their movement was observed, i.e., whether they could move through the rubber layer. All the tested bacteria were unable to move through the rubber layer (Fig. 5) demonstrating, for the first time, that the mechanical defense of latexes could *per se* suffice to defend the plants from bacterial infections, or at least, that the barrier could retard their movement.

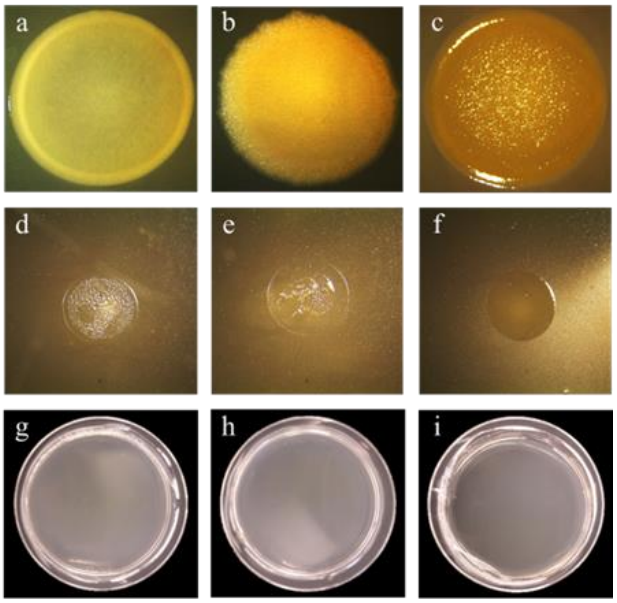

Fig. 5. Antibacterial effect of rubber against bacterial pathogens. a, *Pseudomonas fluorescens*. b, *Pseudomonas putida*. C, *Pseudomonas viridiflava*. The first three images represent the negative control consisting of the bacteria growth in Mueller-Hinton agar (Nutrient agar 2 for *P. viridiflava*) after 24 h; d, e, and f: the same bacteria growing on top of the rubber layer. The bacteria are not able to penetrate the layer and grow in the agar media; g, h, and i: growth control for the rubber treatment. The rubber layer with bacteria on top was removed and the plates incubated for 24 h to reveal possible bacterial growth.

In the case of fungi (*Botritys cinerea* and *Alternaria altenata*), a similar phenomenon, with some differences, was observed. When testing the previously described sealing effect, the layer did not indefinitely block the spreading of the fungal growth and after some time, the fungi were able to penetrate it. However, the movement was definitely delayed so that when the fungi were placed on top of the rubber layer, they were eventually able to break through it but at a very slow rate. In addition, when the diameter of colony submitted to the rubber treatment was compared to the negative control, the radial growth was significantly reduced (Fig. 6A). A possible complementation between polyisoprenes and small molecules in latex was

tested by supplementing the rubber slurry with latex. Results revealed a significant decrease in the radial fungal growth compared to that observed with rubber alone (Fig. 6B).

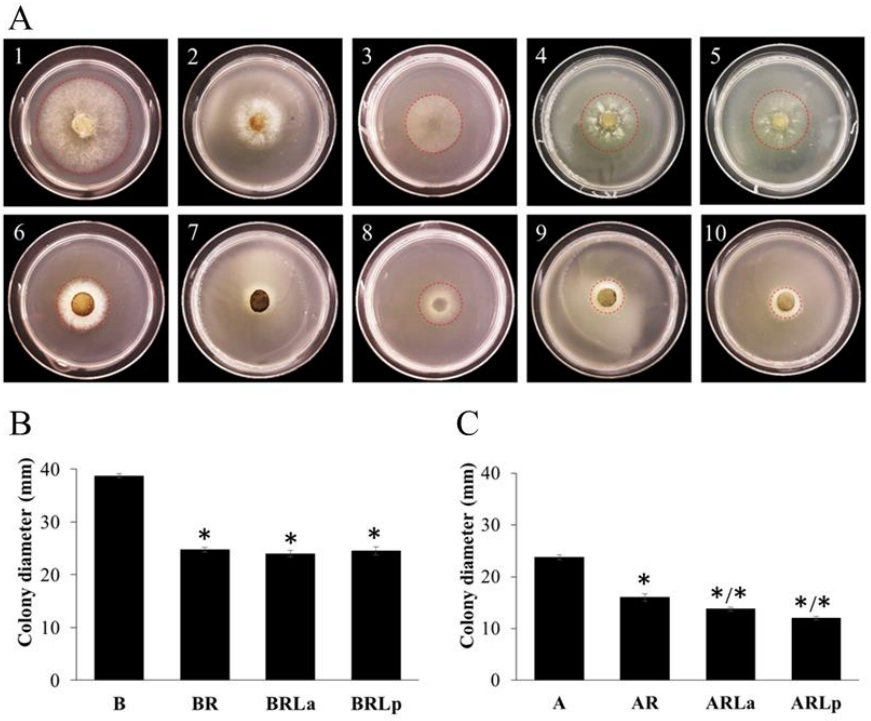

Fig. 6. Antifungal activity of rubber and rubber plus latex against *Botrytis cinerea* and *Alternaria alternate*. (A) Radial growth of *Botrytis cinerea* and *Alternaria alternate* in control and treated medium at 48 h. 1, Growth control of *B. cinerea* growing on PDA medium. 2, *B. cinerea* growing on PDA medium covered with a rubber layer. 3, Mycelium of *B. cinerea* in the agar medium after removing the rubber layer. 4, *B. cinerea* growing on PDA medium covered with a rubber layer combined with 200 μg/mL *E. palustris* latex 5, *B. cinerea* growing on PDA medium covered with a rubber layer combined with 200 μg/mL *E. glareosa* latex. 6, Growth control of *Alternaria alternata* growing on PDA medium. 7, *A. alternata* growing on PDA medium covered with a rubber layer. 8, Mycelium of *A. alternata* in the agar medium after removing the rubber layer. 9, *A. alternata* growing on PDA medium covered with a rubber layer combined with 200 μg/mL *E. palustris* latex 10: *A. alternata* growing on PDA medium covered with a rubber layer combined

with 200 µg/mL *E. glareosa* latex. (B) Antifungal activity of rubber and rubber supplemented with latex at 48 h against *B. cinerea*. The values represent the mean (n = 4) ± standard error. The stars represent significative differences between the treatment and the control ($p \leq 0.05$) in a Dunnett-test. B: growth control of *Botrytis cinerea*; BR: *B. cinerea* growing medium covered with a rubber layer, BRLa, *B. cinerea* growing medium covered with a rubber layer supplemented with latex of *E. amygdaloides*, BRLp, *B. cinerea* growing medium covered with a rubber layer supplemented with latex of *E. palustris*; (C), Antifungal activity of rubber and rubber supplemented with latex at 48 h against *Alternaria alternata*. A: growth control of *Alternaria alternata*, AR: *A. alternate* growing medium covered with a rubber layer, ARLa: *A. alternata* growing medium covered with a rubber layer supplemented with latex of *E. amygdaloides*; ARLp: *A. alternata* growing medium covered with a rubber layer supplemented with latex of *E. palustris*. The values represent the mean (n = 4) ± standard error. The stars represent significant differences between the treatment and the control, a star after a slash indicates significant differences between the treatment and BR ($p \leq 0.05$) in a Dunnett-test. Fungal pathogens are able to penetrate the mechanical barrier of rubber, however, a retardation effect of the fungal proliferation is observed.

In the metabolomics analysis, all the tested *Euphorbia* latexes collected from various locations exhibited a very high amount of cycloartanols together with polyisoprenes. Unlike bacteria, the tested fungi were eventually able to penetrate the rubber barrier albeit after an initial delay. Being the major and selectively concentrated metabolites in latex, cycloartanols were assayed for fungicidal activity. For this, 24-methylenecycloartanol, a cycloartanol isolated from *Euhporbia* latexes was tested for its fungicidal activity and compared with that of other triterpenes and steroids that are commonly found in leaves and roots.

The minimum inhibitory concentration (MIC) values of 24-methylenecycloartanol and other common steroids and terpenoids including β-sitosterol, α-amyrin, β-amyrin, ursolic and oleanolic were determined against *B. cinerea*. With the exception of latex-specific 24-methylenecycloartanol, none of the compounds showed inhibition at the

tested concentrations. Moreover, some of the common steroids or triterpenoids displayed a growth- promoting effect on the fungus. For example, ursolic acid showed a strong growth-promotion effect in liquid suspensions of fungi 64 h after inoculation (Supplementary Fig. 2). It is worth to mention that there was even higher growth at higher concentration (2 mg/mL) of this metabolite. This suggested that 24-methylenecycloartanol plays a specific role in the latex. Interestingly, though the isolated compound displayed a mild inhibition against *B. cinerea* in the range of 500–1000 μg/mL, in latexes this occurred in concentrations as high as 11 000 μg/mL.

It was thus assumed that metabolites assisted the rubber barrier. Presumably, the hyphae of fungi that survived after penetrating the mechanical rubber barrier would then come in direct contact with the latex inside the laticifer that contains active metabolites in higher concentration than in the coagulated layer. This is where 24-methylenecycloartanol, by far the most abundant compound in the latexes, could act on the fungi. These results reveal the existence of a systemic defense mechanism with a synergistic effect between rubber and cycloartanol and highlights how this allows latexes to deal with the threat of a large number of enemies with a very limited number of compounds. Rubber is able to protect the plants from a wide range of pathogens and herbivories simply due to a mechanical effect which is complemented with a chemical defense in the form of specific compounds such as cycloartanols, which act on the residual herbivores or pathogens. Moreover, below the film of coagulated rubber, a high concentration of bioactive metabolites produced by induction will accumulate as a response to the aggression (Krstić et al. 2016), thus combining mechanical and chemical effects in one single barrier.

However, the MIC value of 24-methylenecycloartanol is too high for an active fungicide. This low activity could be explained partially by its low solubility in the aqueous conditions of the test. Thus, it seemed likely that some component/s in the chemical matrix of the latexes acted as a solubilizing agent, avoiding their precipitation. To test this, two solutions, one containing only 20 mg of the isolated 24-methylenecycloartanol and another with poly-*cis*-1,4-isoprene were prepared and then dried out. When the solution containing only cycloartanol was dried, the compound

crystalized, whereas the mixture with polyisoprene formed a layer coating the wall of the tube and the sterol was evenly distributed throughout the layer. This experimental result suggested the potential of rubber as a dispersing agent for lipophilic metabolites, in this case allowing the distribution of the cycloartanol in latexes to increase its efficiency as a defense mechanism. Thus, the results the tests showed that the triterpene-rubber dispersion and rubber films and their combination with latexes acted as a well-designed system against bacterial and fungal microorganisms. In this system, the rubber particles acted as a carrier and disperser of phytosterols and triterpenes during latex exudation and throughout the whole production of a sealing film after coagulation. Therefore, the complementation between the mechanical defense (rubber coagulation) and chemical defense (specialized metabolites) results in a polymeric film in which embedded metabolites are evenly distributed in the whole film to increase its effectiveness over the protected area. In addition to the fungicidal activity and function as a dispersion agent, triterpenes might have other functions. They could contribute to the polymeric structure of polyisoprene to strengthen the defensive barrier, as may be deduced from other related experiments (Mironenko et al. 2016). However, it has not been possible to understand the specificity of the synergy between latex and cycloartanol among many other available steroids and triterpenoids.

Moreover, it also suggests that the basic defense system of latexes due to mechanical features combined with selectively secreted metabolites hardly vary when submitted to environmental changes. Even if this were insufficient to avert fungal infections or fully stop herbivory, the primary latex barrier could provide immediate protection while specific (inducible) defense mechanisms are activated. As an advantage over normal induced responses, the defense system in latexes is much faster, producing a build-up of active compounds in just a couple of minutes (Konno 2011; Agrawal and Konno 2009). Based on the experiments in this study, plant latexes are proposed to play a role as a general primary defense using mechanical (e.g. stickiness and coagulation), chemical (constitutive metabolites) and biochemical responses (specific enzymes) (Konno 2011; Agrawal and Konno 2009; Salomé-Abarca et al. 2019) in a coordinated manner (Fig. 7).

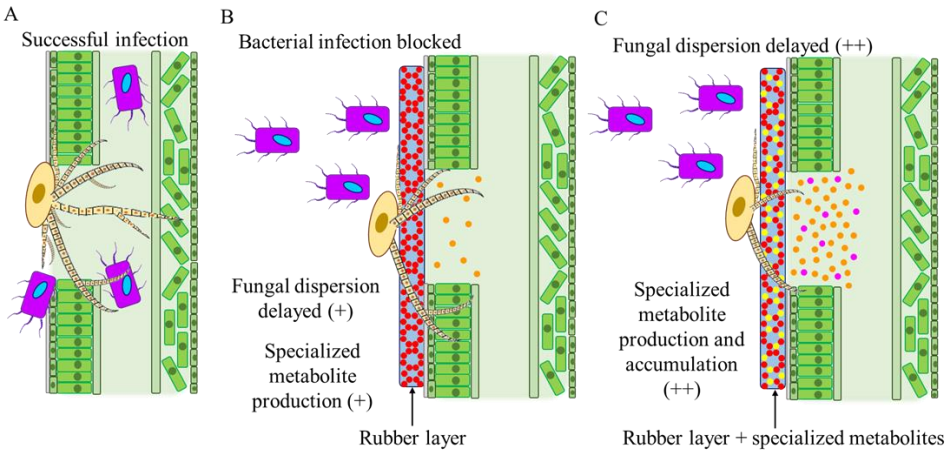

Fig. 7. Chemo-mechanical defense system in latex against general fungal and bacterial pathogens. (A) Open wound in leaves tissue, pathogenic microorganisms are free to enter and cause potential infections. (B) Plant wound covered with a rubber latex after latex coagulation, the mechanical barrier is sufficient to avoid bacterial infections, but only decreases the fungal spread speed. (C) Plant wound covered with a polymerized layer of rubber after latex coagulation is reinforced by specialized metabolites, the fungal pathogens grow and disperse at a lower rate while the induced chemical defense system is activated in the laticifer.

3. Conclusions

Regarding the questions formulated at the beginning of the study: Are the latexes from different species chemically similar? To what degree do environmental factors affect the chemical variation of the latexes? Do specialized metabolites exert their biological activity individually or do they act in a complementary or even synergistic manner with other latex components? If so, how do they interact? It can be conclude that the metabolomes of *Euphorbia* constitutive latexes are well differentiated through species, however, their possess certain degree of chemical similarity, which resulted in their considerably lower metabolic variation across geographical locations as compared to other plant organs such as leaves and roots. This is determined mainly by terpenoids, and it indicates a semi-

conserved constitutive chemical selection in these species. Further, this semi-conserved chemical selection provides to latexes a more stable and less variable effect against generalist herbivores like *M. brassicae*. Moreover, independent metabolic components such as polyisoprenes and terpenoids provide specific types of protection to their bearing plants, specifically mechanical and chemical defense, respectively. These defense features complement each other resulting in a polymeric film in which embedded metabolites are evenly distributed in the whole film to increase its effectiveness over the protected area. The regularity and complementation of latex components suggests that it plays a complementary role in the plant defense system in conjunction with other plant mechanisms such as inducible responses.

4. Experimental

4.1. Plant material and collection

Leaves, roots and latexes of *Euphorbia glareosa* Pall. Ex M. Bieb., *Euphorbia amygdaloides* L. and *Euphorbia palustris* L. were collected in several locations in Serbia: Deliblatska peščara, Zagajička brda, Titelski breg, Avala, Mali Jastrebac, Kosmaj, Borča, Čenta and Šajkaš, at 44°56'41.44"N 21°4'29.52"E, 44°55'48.29"N 21°11'51.68"E, 45°13'23.92"N 20°13'45.03"E, 44°41'11.41"N 20°30'53.20"E, 43°23'3.51"N 21°36'47.97"E, 44°28'28.45"N 20°34'28.10"E, 44°54'48.34"N 20°26'32.51"E, 45°5'58.15"N 20°22'42.06"E, and 45°15'13.14"N 20°6'31.21"E, respectively in June, 2017. The plant materials were identified by Pedja Janaćković and voucher specimens are deposited at the Herbarium of the Botanical Garden "Jevremovac" University of Belgrade, Belgrade, Serbia (Voucher numbers: *Euphorbia glareosa* (BEOU17303), *Euphorbia amygdaloides* (BEOU17306), and *Euphorbia palustris* (BEOU17304). Latex samples were collected by slicing plant stems with a sterile razor blade. Then, approximately 1 mL of latex was collected in a sterile 2 mL-microtube containing 400 µL of MeOH. The samples were stored at -20 °C until they were freeze-dried. Leaf and root samples were manually collected from the plant and placed into plastic hermetic bags with

silica gel. This material was stored at -20 °C until processed. For this, the leaf and root samples were ground with liquid nitrogen and then freeze-dried. Dry leaf (10 g) and root (5 g) powders were extracted with methanol with sonication during 15 min. The solvent was evaporated with a rotary evaporator and the extracts were taken to total dryness with a speed-vacuum dryer.

4.2. Organisms

Larvae of *Mamestra brassicae* were kindly provided by Pieter Rouweler from the Entomology department at Wageningen University. *Pseudomonas putida* (NCCB26044), *Pseudomonas fluorescens* and *Pseudomonas viridiflava* were kindly provided by Dr. Paolina Garbeva (Kurm et al. 2019). *Alternaria alternata* (CBS 102.47) strain was purchased from the collection of the Westerdijk Fungal Biodiversity Institute, and *Botrytis cinerea* was kindly provided by Dr. Jan van Kan (Van Kan et al. 2017).

4.3. HPTLC-DART-MS

For thin-layer chromatography, latexes, leaf and root methanol extracts were prepared at a final concentration of 2 mg/mL. An automatic TLC sampler (ATS 4) (CAMAG, Muttenz, Switzerland) with a 25 µL Hamilton syringe was used to apply 30 µg of all of the samples as 6 mm bands on 20 × 10 cm HPTLC silica gel plates (60 F254), (Merck). The samples were applied at 20 mm from the lateral edges and 10 mm from the bottom of the plate. The distance between bands was 10 mm resulting in 18 tracks per plate. The chromatographic development was performed in an automatic developer (ADC2) (CAMAG, Muttenz, Switzerland). The samples were separated with a mixture of toluene–ethyl acetate (8:2, v/v). The saturation time was 20 min and humidity was set to 37 % using a saturated $MgCl_2$ solution. The solvent migration distance was 75 mm from the application point. The HPTLC system was controlled by Vision Cats software.

For HPTLC-DART-MS analysis, each track of the plate was cut in 5 mm-width strips using a smart glass plate cutter (CAMAG, Muttenz,

Switzerland). The HPTLC strips were individually placed on a motorized rail to be moved to the ionization region. The plates were ionized with a DART ion source (Ion Sense, Tokyo, Japan) using helium gas (purity of 99.999%) at 450 °C and 3 L h^{-1}. The plate scan speed was 0.2 mm s^{-1} and it was controlled with the DART control software (Ion-Sense). The distance from the ion source to the plate was 1.5 cm. The detection was performed with an AccuTOF-TLC (JEOL, Tokyo, Japan) in positive ion mode. The TOF-MS was set with a peak voltage of 800 V and a detector voltage of 1,900 V.

4.4. ^{1}H NMR analysis

Five mg of freeze-dried latexes were re-suspended in 1 mL of CH_3OH-d_4 containing 3.93 mM hexamethyldisiloxane (HMDSO) as the internal standard, and ultrasonicated for 20 minutes. For leaf and root samples, 5 mg of the MeOH extract were dissolved in 1 mL of CH_3OH-d_4. All the solutions were centrifuged at 13000 rpm for 10 min, and 300 µL of the supernatant were transferred into 3 mm-NMR tubes. The ^{1}H NMR analysis was carried out with an AV-600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), operating at proton NMR frequency of 600.13 MHz. For internal locking CH_3OH-d_4 was used. All ^{1}H NMR consisted of 64 scans requiring 10 min and 26s as acquisition time using the parameters: 0.16Hz/point, pulse width (PW) = 30° (11.3 µs), and relaxation time of 1.5 s. A pre-saturation sequence was used to suppress the residual water signal using low power selective irradiation at H_2O frequency during the recycle delay. The FIDs were Fourier transformed with exponential line broadening of 0.3 Hz. The resulting spectrums were manually phased and baseline corrected, and calibrated to HMDSO at 0.06 ppm using TOPSPIN V. 3.0 (Bruker).

4.5. Gas Chromatography coupled to mass spectrometry (GC-MS)

Dried latexes (5 mg) were extracted with 1 mL of chloroform. The extract was taken to total dryness with a speed vacuum dryer. The dried extracts were re-dissolved with 100 µL of pyridine by ultrasonication for 5 minutes. To this, 100 µL of BSTFA + TMCS (99:1, Supelco) were added and the

solutions were heated at 80 °C for 50 min. The solutions were then centrifuged at 13 000 rpm for 10 min and the supernatants were transferred to micro-inserts for GCMS analysis on a 7890A gas chromatograph equipped with a 7693 automatic sampler coupled to a 5975C mass single-quadrupole detector (Agilent, Folsom, CA, USA). Separation was performed on a DB5 GC column (30 m x 0.25 mm, 0.25 µm thickness, JW Science, Folsom, CA, USA) with helium (99.9% purity) as the carrier gas at a flow rate of 1 mL/min. The initial oven temperature was 100 °C for 2 min, and then ramped at 10 °C/min to 270 °C, held for 1 min, ramped again to 290 °C at 5 °C/min for 15 min and then to 300 °C at 5 °C/min and held for 3 min. The injector was set at 280 °C and 1 µL of each sample was injected in t splitless mode. The interface temperature was 280 °C, and the ion source and quadrupole temperature of the mass detector were 230 °C and 150 °C, respectively. Ionization energy in EI mode was 70 eV and peaks were identified by comparison of the ion spectra with the NIST library (version 2008).

4.6. Liquid chromatography coupled to mass spectrometry (LC-MS)

Five milligrams of each latex sample were individually re-suspended in 1 mL of a methanol:water solution (80:20, v/v) and ultrasonicated for 15 min. The resulting extracts were diluted in a 1:10 (v/v) ratio to reach a final concentration of 0.5 mg mL^{-1}. The samples were filtered with 0.20 µm membrane filters. Samples were analyzed with an Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 µm; Waters). Samples were eluted with a gradient of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) starting at 10 % B (0 – 30 min), 100 % B (30 – 35 min), 10 % B (35 – 40 min) at 40 °C at a flow rate of 0.3 mL min^{-1} The MS detection was performed on a QTOF mass spectrometer (Bruker Impact HD) equipped with an electro-spray ionization (ESI) source. The capillary voltage was 4000 V and the drying temperature was 350 °C at 6 L min^{-1}. The samples were analyzed in negative and positive mode in the range of 50-1200 *m/z*. Data acquisition, alignment, peak picking and neutral losses calculations were performed using Progenies QI software version 2.3 (Nonlinear Dynamics a Waters company, Newcastle, UK). Quality control (QC) samples consisted of

a blend of all samples that was injected every five samples. Extraction solvent was injected as a blank. The data was normalized to total intensity and filtered by deleting mass features detected in blank samples at higher response levels than in latex samples. Data filtering resulted in 8015 mass features for data acquired in positive mode and 2064 features in the negative mode. Compounds were identified by comparison of their exact mass with the Dictionary of natural products. A threshold of 10 ppm was set as the mass error for possible matches.

4.7. cis-1,4-polyisoprene solution preparation

Five grams of rubber (Sigma-Aldrich) were cut into small pieces and placed in 100 mL of chloroform. The rubber pieces were left in the solvent 3 hours to swell and then manually stirred every 20 min for 1 min until a semi-clear solution was obtained. The volume was then adjusted to 140 mL and stirred until a clear rubber slurry was formed.

4.8. Microdilution antibacterial assay

The broth microdilution method was used to determine the minimal inhibitory concentration (MIC) of the tested triterpenes according to the Clinical Laboratory Standards Institute guideline. The strains were inoculated on Mueller-Hinton agar (MHA) plates and incubated overnight at 37℃. From the overnight cultures, a single colony was used to inoculate 10 mL of Mueller-Hinton broth (MHB) and incubated at 37℃ under constant agitation (150 rpm). The bacterial suspensions were further adjusted with the addition of MHB to 0.5 of turbidity of the McFarland scale (10^6 CFU/mL). Parallel, the compounds were dissolved in DMSO and two fold dilutions were done to reach final concentrations in the well starting from 512 μg/mL to 16 μg/mL, also diluted in MHB with a volume of 100 μl in each well. Then, each well was inoculated with 50 μl of the 0.5 McFarland bacterial suspensions and incubated for 24 h at 30 ºC. The final concentration of DMSO in the well was 5 %, which was also used as a negative control. Spectinomycin at 100 μg/mL in the well was used as a positive control. The bacterial growth was measured

by optical density at 600 nm in a well microtiter plate reader (SPARK 10M, TECAN). The MIC value was defined as the lowest concentration of a compound that completely inhibited the bacterial growth at 24 h. All experiments were performed in triplicates. For *P. viridiflava* the inoculations were done nutrient agar 2 plates and the assays were carried out in nutrient broth 2 at 28 ºC.

4.8.1. Antibacterial assay

The bacterial strains were prepared in the same manner as in the microdilution method. The agar plates with treatments were prepared by filling 45 mm Petri dishes with 7 mL of MHA (Nutrient agar 2 for *P. viridiflava*); after medium solidification, 3 mL of rubber solution was poured on top of the medium and left to dry out for 2 h in a fume hood resulting in a homogeneous rubber layer of c.a. 0.05 mm of thickness. Finally the plates were exposed for 10 min to UV light for sanitation. The negative controls were MHA plates without rubber covering. Four replicates were performed for treatments and controls of *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas viridiflava*. The bacterial growth was evaluated at 24 h and 48 h. To determine whether bacteria was unable to pass through the rubber layer to the agar, the rubber layer from the inoculated treatment plates was removed and the plates were incubated for 24 h at 28 ± 2 °C. Plates were then inspected for the appearance of bacterial colonies at the inoculation points.

4.9. Antifungal assays

The treated plates were prepared similarly to those used for antibacterial assays, but using potato dextrose agar (PDA). Seven millimeter agar plugs with *Alternaria alternata* and *Botrytis cinerea* were placed on top of the rubber layer of the treated plates. The negative controls were PDA plates without a rubber layer and PDA plates just with rubber and latex. To observe if the fungi grew over the rubber layer or penetrated it, the layer was manually removed from the plate. Four replicates were performed for each

fungal strain and their growth and colony diameter was measured at 48 h. In the case of the rubber and latex combination, *E. palustris* and *E. myrsinites* were chosen as models because of their very different triterpenoid profile. Five milligrams of all samples of each species were mixed to get a representative sample of each latex. The latex was then dissolved in the rubber solution to reach a concentration of 200 µg/mL. The plates were dried in the same way as in previous experiments.

4.10. Minimum inhibitory concentration (MIC)

24-methylenecycloartenol, β-sitosterol, as representatives of different steps of the phytosterol pathway, α-amyrin, β-amyrin, ursolic and oleanolic acids, as representatives of different steps in the triterpene pathway were selected to be tested against *Botrytis cinerea*. The compounds were dissolved in methanol and tested in two-fold dilution series from 2000 µg/mL until 62.5 µg/mL. The spore solution was adjusted to 2.5 x 10^5 spores/mL in the well and the final concentration of methanol in the well was 5 %. The positive control consisted of nystatin in the same range of concentrations, and the negative control consisted in media with final concentration of 5 % of methanol in the well. The MIC value was defined as the minimum concentration in which there was visible total inhibition of the fungal growth at 16 h. The plates were examined under a stereoscopic microscope at 16 h-intervals to observe any further effects on the fungal growth.

4.11. Anti-herbivore activity

The diet was prepared by mixing 28 g of agar, 160 g of cornflower, 50 g of beer-yeast, 2 g of sorbic acid, 1.6 g of methyl-4-hydroxybenzoate, 8 g of ascorbic acid and 0.1 g of streptomycin per liter of water. The ingredients were added to warm water with continual stirring. The diet (15 mL) was placed in individual plastic containers and left to solidify at room temperature. For treatments, the dry methanol extracts were re-suspended in 2 mL of ultrapure water and ultrasonicated for 10 min (2X). These were added to the diets while they were still semiliquid, manually mixed and left to

solidify at room temperature. The treatments consisted of methanol extracts of latexes, leaves and roots. From the ten samples from each location of each species, three random samples were mixed to form one composed sample. Thus, 3 composed samples from each region of each plant species were obtained for the three tissues resulting in 9 replicates for each species of each tissue and three replicates for different locations. The final concentration of all the treatments was 200 μg/mL. The negative control consisted of ultrapure water and the positive control was a 200 μg/mL concentration of abamectin. The weight of the larvae was recorded after five days and the weight of treated and untreated (negative control) were compared.

4.12. Data processing and multivariate data analysis

The NMR spectra were bucketed using AMIX 3.9.12 (Bruker BioSpin GmbH, Rheinstetten, Germany). Bucket data was obtained by spectra integration at 0.04 ppm intervals. Peak intensity of individual peaks was scaled to total intensity and recorded from δ 0.20 to 10.02. Because of the residual signals of D_2O and CH_3OH-d_4, regions of δ 4.75 – 4.9 and δ 3.28 – 3.34 were excluded from the analysis, respectively. Multivariate data analysis was performed using SIMCA P (v.15, Umeå, Sweden). Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed for ^{1}H NMR and LC-MS data. For PCA analysis data and PLS-DA were scaled using the unit variance (UV) method.

To assess the effect of the geographical region on chemical diversity, a soft independent model of class analogy (SIMCA) analysis was performed using geographical origin and plant species as PCA-classes separately in each plant species. The distances to the model (DModx) values were calculated setting each plant species as a PCA-class in the species effects model, and the same geographical region in each species for the geographical origin effect in tissues. Data was scaled using the UV method and the DModx values were transformed to their corresponding logarithm values. The logarithmic averaged DModx values (n = 30) ± standard error of each model were used as a measure of the strength of each factor in the chemical homogeneity of the samples.

In order to obtain and compare the total correlation of the effects of the geographical origin of the species on the chemical composition of the samples, PLS-DA modelling using UV scaling was also performed on each individual data set. The averaged Q^2 from the permutation test (100 permutations) were used as a measure of the strength of the effects of the species and geographical origin on the chemical profile differences among the different plant tissues.

For the antifungal bioassays, the radial growth of the treatments were compared to their corresponding control by a Dunnett test setting the control sample as control for the comparison of the treatments to the control, and setting the rubber treatment as control for its comparison to the latex supplemented treatments (α = 0.05). The anti-herbivory activity data variance, homogeneity, and mean comparison was done with a type 2 ANOVA and the mean comparison was performed with a Least-square means test (α = 0.05) after log transformation of the data using R software (V 1.1.456).

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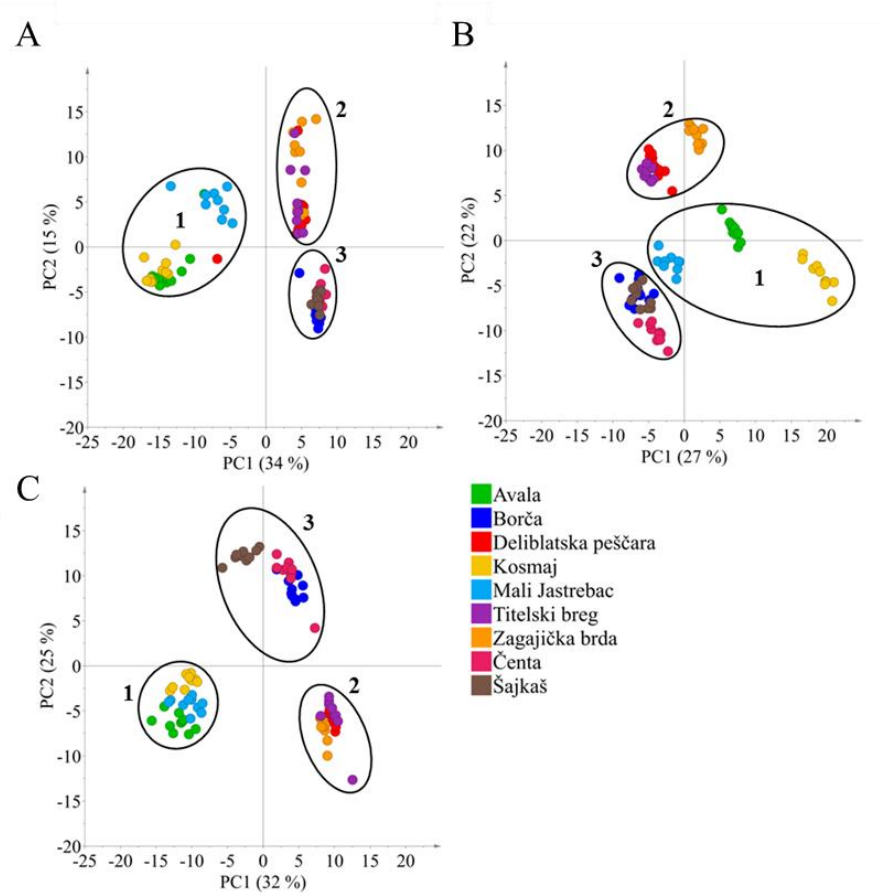
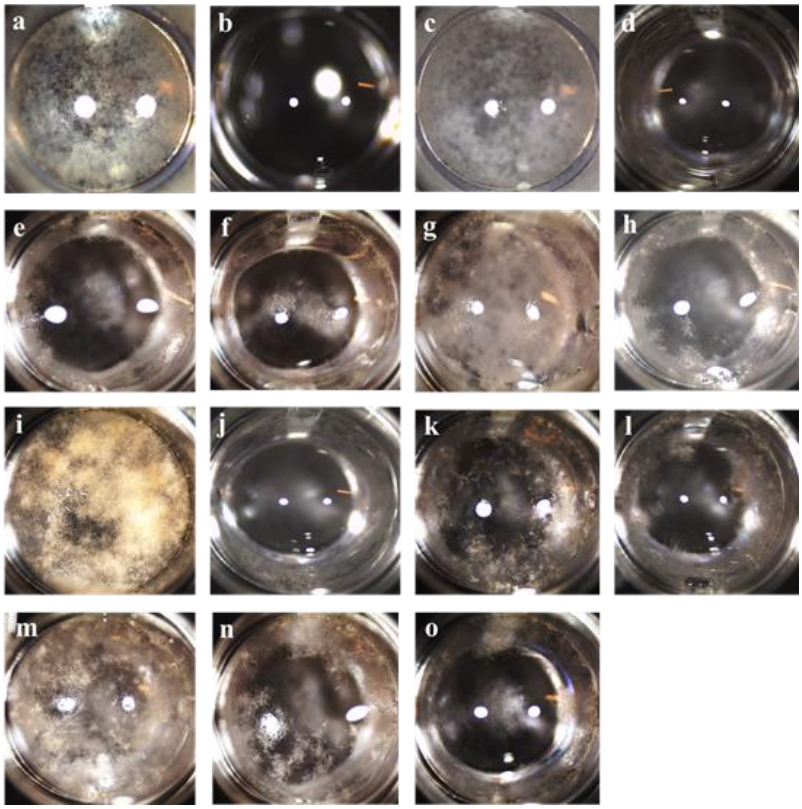
Chapter 5

Supplementary figures



Supplementary Fig. S1. Principal component analysis of latex, leaves, and roots of three *Euphorbia* species collected a different locations of Serbia. A, latex, B, leaves, C, roots. 1, *Euphorbia amygdaloides*, 2, *Euphorbia glareosa*, 3, *Euphorbia palustris*. The separation of the samples by geographical origin is much more evident in leaves and much less evident in latexes.



Supplementary Fig. S2. Effect of triterpenes over the growth of *Botrytis cinerea*. a, Nitastine [2 mg/mL], b, Nitastine [62.5 µg/mL], c, 24-methylenecycloartanol [2 mg/mL], d, 24-methylenecycloartanol [62.5 µg/mL], e, α-amyrin [2 mg/mL] f, α-amyrin [62.5 µg/mL] g, β-amyrin [2 mg/mL] h, β-amyrin [62.5 µg/mL] i, oleanoic acid [2 mg/mL], j, oleanoic [62.5 µg/mL], k, ursolic acid [2 mg/mL], l, ursolic acid [62.5 µg/mL], m, β-sitosterol [2 mg/mL], n, β-sitosterol [62.5 µg/mL], o, negative control.