Phytohormone Profiling reveals fungal Signatures and strong Manipulation of Infection Cycle in the *Gymnosporangium juniper-virginianae* Dual-Host Plant System

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Phytohormone profiling reveals fungal signatures and strong manipulation of infection cycle in the *Gymnosporangium juniper-virginianae* dual-host plant system

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Abstract: The aim of this work was to examine the function of phytohormones in the pathogenesis of cedar-apple rust, a fungal disease caused by *Gymnosporangium juniper-virginianae* Schwein. on Eastern red cedar (*Juniperus virginiana* L.) and crabapple trees (*Malus* spp. Mill.). Control cedar branchlets, gall tissues, fungal telial horns, as well as healthy and infected apple leaves were collected throughout fungal and plant development and used for profiling endogenous cytokinins (CK) and abscisic acid (ABA) by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS). Phytohormone composition implicates cytokinin involvement in the development of rust infection. Moreover, increased levels of total CKs, as well as the unique profiles of sporulating galls on cedar trees, telial horns, and infected apple leaves, dominated by *cis*-Zeatin (cZ) type CK, suggest that the fungus can synthesize hormones to facilitate the infection process. Distribution of ABA in the fungal and plant tissues indicates an important function of this stress hormone in regulating rapid changes in osmotic pressure during teliospore production by the galls. This study of the cedar-apple rust disease cycle is the first elucidation of phytohormones profiling between a pathogenic fungus and the attacked plant in a dual-host infection system.

Key words: cedar-apple rust, gall-forming fungi, phytohormones, cytokinin, *cis*-Zeatin, abscisic acid

39 Introduction

40 Cedar-apple rust (*Gymnosporangium juniper-virginianae*) is an obligate, biotrophic,
41 heteroecious fungal parasite that infects apple tree leaves and fruits, while wind-born spores
42 travel and infect cedar trees, forming galls (Anikster 1985; Kolmer et al. 2009). The infected
43 apple trees are weakened, and rust growing on fruits significantly reduces their market value.
44 Consecutive years of infection may result in the failure to yield or even in death of the tree. The
45 fungus is a substantial threat to apple orchards since no effective preventative measures are
46 available. Currently, all cedar trees in a few kilometer radius must be destroyed, or costly and
47 often ineffective chemical treatments utilized (Ziems 2009). Moreover, the destruction of cedar
48 trees adjacent to orchards is not a sustainable approach as it impacts both the environment and
49 ecosystem. Cedar trees often act as a windbreak, preventing erosion in their vicinity (Ziems
50 2009; Gauthier and Heisdorffer 2015).

51 Figure 1 presents the life cycle of cedar-apple rust that involves two plant host species.
52 The fungus overwinters in spherical galls on cedar tree branchlets (C1). Early spring rains cause
53 horn-like structures (telial horns) to extrude from galls (C2). The horns become jelly-like and
54 swollen when they absorb water, while they dry to dark brown threads (C6) between the rain
55 periods. This swelling and drying of telial horns may occur quickly depending on the
56 environmental factors and can happen up to 10 times during the season. The telial horns contain
57 thousands of two-celled teliospores (C3). Each swelling/drying cycle causes the horns to be
58 pushed out further and expose more teliospores, until the supply is exhausted. Teliospores
59 germinate to produce a germ tube (basidium) (C4) from each cell. Four basidiospores are

produced on each basidium. At optimum temperatures, basidiospores (C5) are produced within 4 hours of the horns absorbing water. Basidiospores are forcibly discharged into the air immediately after being formed and can be carried long distances with the wind. After all the teliospores have been exposed and all basidiospores released, the gall completes its part of the life cycle, begins to dry up and eventually dies (C7). Basidiospores that land on young apple leaves or fruits can germinate and infect the tissue if a film of water is present for an adequate period of time. One to two weeks after the infection, orange pustules (pycnia) containing pycniospores appear on the upper side of apple leaves (A1). One to two months after the appearance of pycnia, the rust produces brownish clusters of threads or cylindrical tubes (aecia) on the underside of the leaves or on fruits (A2). The aecia produce aeciospores (A3), which are released into the air during dry conditions in late summer. Aeciospores that land on young cedar twigs germinate, infect, and cause gall formation in the following year. Generally, the gall matures and produces teliospores in the second year after infection, thereby continuing the disease cycle. Because most galls produce teliospores for only one season, a new crop of galls is required each year if infection of apple trees is to occur.

There are an estimated 7000 species of rust fungi that infect an array of vascular plants from ferns to monocot and dicot species. Rust species have evolved specifically with the plant hosts they infect, colonize, and upon which they reproduce (Kolmer et al. 2009). Host resistance mechanisms include race non-specific resistance and race-specific resistance. Race non-specific resistance can be attributed to a group of minor genes that provide defense against several pathogens (Sekhwal et al. 2015). For instance, in maize a cluster of resistance genes called the

rp1 complex have been shown to confer a race non-specific resistance response to rust (Hulbert 1997). In contrast, race-specific resistance involves plant resistance genes that recognize the pathogen avirulence gene effectors, triggering the plant defense response (Sekhwal et al. 2015). Infection occurs when the plant cannot recognize the rust, as either the rust isolates lack the specific avirulence genes, or host plants lack the specific resistance genes (Huang et al. 2003).

Plants developed several defense mechanisms against pathogens. Primarily, constitutive defenses provide resistance barriers like cell walls, waxy epidermal cuticles, and bark. Secondly, inducible defenses that stem from resistance genes, lessen or mediate pathogenic infection by producing toxic chemicals such as pathogen-degrading enzymes. Rapid local programmed cell death or hypersensitive response sequester infected cells to partition off infected tissue, restricting resource allocation. Additionally, plant production of defense response phytohormones, including jasmonic acid, salicylic acid and volatile organic compounds can create an environment unfavorable to pathogen invasion or signal the regulation of other defense factors (Freeman et al. 2008; Arya et al. 2014).

The mechanisms that involve hormone manipulation are adapted in many different pathogenic systems to keep the host plant alive throughout parasitic life cycles (Tooker et al. 2008; Straka et al. 2010; Zhang et al. 2017). Biotrophic fungal pathogens often use phytohormones such as cytokinin to successfully overcome plant defense barriers (Chanclud et al. 2016; Chanclud and Morel 2016; Morrison et al. 2017; Patkar and Naqvi 2017). Cytokinins (CKs) are a group of growth regulators that have a key function in plant physiology. CKs are essential for cell division and differentiation in plants, thus they are most abundant in the

proliferating tissues. They play a role in the delay of senescence and the mobilization of nutrients to the sink organs (Argueso et al. 2009; Werner and Schmülling 2009). Interestingly, CKs can be produced by other organisms and used to manipulate the growth of plants. This inter-kingdom hormone messaging may result in production of food, shelter or create a symbiotic relationship between the organism and the plant (Stirk and van Staden 2010). Fungal derived cytokinins can assist in side stepping a hypertensive response and delaying plant senescence through the powerful ability to mobilize nutrients towards the sites of application (Walters and McRoberts 2006; Shigenaga and Argueso 2016). CKs produced by the gall forming fungal pathogen *Ustilago maydis* (Persoon) Roussel cause differentiation of the phytohormone profiles in the infected and uninfected tissues and are used to lessen plant ability to defend itself against the invasion (Morrison et al. 2017). It was previously reported, that increased CKs levels affect sugar and amino acid distribution around the site of infection to favor the fungus growth (Chanclud et al. 2016).

Abscisic acid (ABA) is a hormone synthesized by plants under stress conditions and its function is usually linked with defense mechanisms. The results of previous studies often support a negative role of ABA in plant immunity (Cao et al. 2011). It has been suggested that ABA has a role in increasing host susceptibility to pathogens and assists in gall formation (Mauch-Mani and Mauch 2005). ABA has been proposed to have a stimulatory effect on gall formation and development on hackberry trees (Straka et al. 2010). An antagonistic relationship between ABA and salicylic acid which acts as a crucial plant defense mechanism has also been reported (Xu et al. 2013).

Our work investigates the CK and ABA phytohormone profiles within the unique cedar-apple rust system throughout the life cycle of the fungus and its host plants, to better understand the nature of the potential cross-species biochemical interactions. Based on the composition of phytohormones observed during rust pathogenesis, we hypothesize that CKs and ABA are involved in the formation and maintenance of galls on cedar trees and play a crucial role in other stages of rust disease development. This is the first report of phytohormones signaling processes between a pathogenic fungus and the attacked plant in a dual-host infection system.

Materials and methods

Tissue Collection

Samples were collected from both host trees grown in the area of Peterborough, Canada, throughout the subsequent stages of rust infection development. The Eastern red cedar (*Juniperus virginiana*) samples included: control, uninfected cedar branchlets (C0), pre-sporulation gall (C1), dry sporulation gall (C6), and post-sporulation gall tissue (C7). Additionally, both wet telial horns (C2) and dry telial horns (C6) were isolated separately from the collected sporulating galls. The crabapple tree (*Malus* spp.) samples included: control, uninfected leaf (A0) and fragments of infected leaves, containing pycnia (A1) on upper, and aecia (A2) on bottom part of the tissue. Bracketed abbreviations of the different tissue types refer to the labeling system used in Figure 1.

All the tested tissue types were collected in triplicates from three different plants and weighed to 0.100 g. Dissected samples were stored at -20° C until further processing.

Hormone Extraction and Purification

A modified protocol described by Quesnelle and Emery (2007) and Farrow and Emery (2012) was used for the CK and ABA extraction. The tissues were suspended in Bielecki #2 buffer (CH₃OH:H₂O:HCOOH [15:4:1, v/v/v]). Samples were spiked with internal standards (144.7 ng of ²H₄-ABA (NRC-PBI, Saskatchewan, Canada)) and 10 ng of each of the deuterated internal standard CKs (OlChemim Ltd., Olomouc, Czech Republic; Table 1), and homogenized in a ball mill (RetschMM300, Retsch GmbH, Haan, Germany; 5min/25 Hz) at 4°C with sterile zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada). The samples were allowed to extract passively overnight (approximately 12 hours) at -20°C. Pellets were removed by centrifugation (Thermo Scientific; Model Sorvall ST16, Ottawa, Canada; 10 min at 5000 RPM), the obtained extracts collected and samples were re-extracted with 1.0 mL extraction buffer at -20°C for 30 min. The pooled supernatants were dried in a speed vacuum concentrator at 35°C.

Extraction residues were reconstituted in 0.200 mL of 1M formic acid (pH 1.4) to ensure complete protonation of all CKs. Each extract was purified on a mixed mode, reverse-phase, cation-exchange cartridge (Oasis MCX, 2cc, 96-well plate; Waters, Mississauga, Canada) using an automated liquid handling protocol (Model 215 SPE System; Gilson Inc., Middleton, USA). Cartridges were activated with 1.0 mL of HPLC grade methanol and equilibrated using 1.0 mL of 1M formic acid (pH 1.4). After equilibration, each sample was loaded and washed with 1.0 mL of 1M formic acid (pH 1.4). ABA and CKs were eluted based on their chemical properties.

ABA was eluted first using 1 mL of HPLC grade methanol. The nucleotide fraction (NTs) was eluted using 1.0 mL of 0.35 M ammonium hydroxide, free bases (FBs), ribosides (RBs), methylthiols (METs), and glucosides (GLUCs) were retained on the column based on charge and hydrophobic properties and, thus, these were eluted last using 1.0 mL of 0.35 M ammonium hydroxide in 60% methanol. All samples were dried in a speed vacuum concentrator at 35°C, and stored at -20°C until further processing.

Nucleotides were dephosphorylated using 3 units of alkaline phosphatase (alkaline phosphatase calf intestine; New England BioLabs, Whitby, Canada) in 1.0 mL of 0.100 M ethanolamine-HCL (pH 10.4) for 12 hours at 37°C. The resulting RBs were brought to dryness in a speed vacuum concentrator at 35°C. Samples were re-constituted in 0.30 mL double distilled Milli-Q water for further purification on a reversed-phase C18 column (C18, 2cc, 96-well plate; Canadian Life Sciences, Peterborough, Canada). Columns were activated using 0.60 mL HPLC grade methanol and equilibrated with 1.20 mL double distilled Milli-Q water. The samples were loaded onto the C18 cartridge and allowed to pass through the column by gravity. The sorbent was washed with 0.60 mL of Milli-Q water and resultant RBs were eluted using 1.0 mL HPLC grade methanol. All sample eluents were dried in a speed vacuum concentrator at 35°C and stored at -20°C until further processing.

Prior to LC-MS/MS analysis, all dried samples were re-constituted in 1.50 mL of starting conditions buffer ($\text{CH}_3\text{COOH}:\text{CH}_3\text{CN}:\text{ddH}_2\text{O}$ [0.08:5.0:94.92, v/v/v] for CKs, and $\text{CH}_3\text{COOH}:\text{CH}_3\text{OH}:\text{ddH}_2\text{O}$ [0.08:5.0:94.92, v/v/v] for ABA).

Hormone Quantification and Analysis by HPLC-(ESI)-MS/MS

Hormones were identified and quantified by HPLC-(ESI)-MS/MS (high performance liquid chromatography - electrospray ionization - tandem mass spectrometry; Agilent 1100 series HPLC (Santa Clara, USA) connected to a 5500 API Mass Spectrometer (AB Sciex, Framingham, USA)). A 20 μL sample volume was injected on a reversed-phase C18 column (Luna, 3 μm , 150 $\times$ 2.1 μm ; Phenomenex, Torrance, USA); all CK samples were analyzed in positive-ion mode (ESI+) and ABA samples were analyzed in negative-ion mode (ESI-). CKs were eluted with an increasing gradient of 0.08% acetic acid in acetonitrile (A) mixed with 0.08% acetic acid in double distilled water (B) at a flow rate of 0.2 mL min^{-1} . The initial conditions were 5% A and 95% B, changing linearly in 17 minutes to 95% A and 5% B. Conditions remained constant for 5 minutes, and then immediately returned back to initial conditions for 18 minutes. ABA was eluted using component A: H_2O with 0.08% $\text{CH}_3\text{CO}_2\text{H}$ and component B: CH_3OH with 0.08% $\text{CH}_3\text{CO}_2\text{H}$, at a flow rate of 0.2 mL min^{-1} . Initial conditions for the ABA fraction were 50% B changing on a linear gradient to 80% B over 8 minutes. This ratio was then held constant for 2 minutes before returning to starting conditions and equilibrating for 8 minutes. The effluent was introduced into the electrospray source (source block temperature of 700°C), and CKs and ABA were identified based on their MRM channels and retention times.

All data were analyzed using Analyst (v 1.5) software (AB Sciex, Framingham, USA), to calculate peak area. Quantification was achieved through isotope dilution analysis based on the recovery of ^2H -labelled internal standards, in accordance with those listed in Table 1.

Results

Full CK and ABA profiles of all the tissue types analyzed in this work are presented in Table 2 and further interpretation of the most significant findings in Figures 2 and 3. Among all the samples collected from cedar trees, sporulation galls contained the highest amount of total CKs (Figure 2). In the apple system, infected leaves contained 2.5 times more of the total CKs than the uninfected leaves.

Mass spectrometric analysis revealed distinctive profiles of cZ (*cis*-Zeatin) type cytokinins among the tested plant and fungal tissues (Figure 3). Within the cedar system, the highest levels of cZ forms were detected in sporulation gall samples. *cis*-Zeatin levels of sporulation galls were composed of active free bases (FB) and precursor nucleotide (NT) forms. On the contrary, post-sporulation galls showed a decrease in overall cZ level and no active FB forms. Wet telial horns, comprised entirely of fungal resting spores (teliospores), contained lower levels of cZ forms than the other analyzed tissues; however, cZ types were the only CKs present in this sample type. FB, RB, and NT forms of *cis*-Zeatin were not detected in the control cedar branchlets and present only in very low quantities during pre-sporulation development. Within the apple system, cZ form levels (FB, RB, and NT) were significantly higher in the rust infected leaves, compared to the healthy, control apple leaves.

Absciscic acid (ABA) was present in great abundance in all eight tissue types (Table 2). Each of the three stages of gall development showed strikingly higher levels of ABA than the cedar branchlet controls. Sporulation galls contained the highest amount of ABA of all the tested tissues, which was over 28 times higher than ABA level detected in cedar branchlet controls.

ABA level was slightly higher in the dry telial horns than in the wet telial horns. ABA was also the only phytohormone found in the dry telial horns. Remarkably, control apple leaves contained 1.5 times higher ABA levels than the infected leaves.

Discussion

The mass-spectrometric examination of a wide range of cytokinin and ABA metabolites showed that phytohormone profiles clearly differ between the two host plants, and also revealed a very unique CK composition that was characteristic for the pathogen samples. The dynamics of cytokinin production during plant infection suggests their strong relationship with the rust lifecycle. We consider two potential mechanisms to explain our findings. The first is that the fungus is able to manipulate host CK biochemical pathway by redirecting CKs produced by plant to facilitate infection, or second, that the pathogen is producing CKs itself which in turn suggests the existence of a CK metabolic pathway within rust.

For both hosts the tissues undergoing sporulation (galls and infected apple leaves) contained the highest levels of total CKs (Figure 2). The sporulating galls are composed of both plant tissue and fungal teliospores. CKs found within these organs included tZ, cZ, and iP forms. Meanwhile, isolated from sporulating galls wet telia that are composed only of teliospores, contained just cZR and cZNT. On the other hand, no active FB fraction and no cZ form CKs were detected in cedar control twigs. In a similar manner, infected apple tissue comprised of pycnia on the upper (adaxial) and aecia on the lower (abaxial) leaf surface where the aeciospore production occurs, had much different hormone profiles than the non-infected control leaves.

Significant shift from tZ to cZ type in hormone profiles during rust infection and disease development suggests that the fungus is excreting hormones using the common or modified mevalonate (MVA) pathway, similar to the one described for *U. maydis*, a fungus infecting corn (Morrison et al. 2015a, 2017) and consistent with the signature CK pathway reported from a large survey of fungal species (Morrison et al. 2015b). On the contrary, in the non-infected control cedar twigs and apple leaves the plant methylerythritol phosphate (MEP) pathway was shown to be more active, resulting in production of the tZ isomers, as well as DZ and iP type CKs (Morrison et al. 2015a).

We propose that the fungal derived cZ type CKs are modifying the host plant nutrient flux by stimulating and maintaining the source-sink relationship to promote a nutrient enriched environment where fungal development is facilitated. Additionally, the virulence of the infection may be modified by CK activity. The reduced virulence of fungal pathogen infection has been linked to the moderation of CK biosynthesis in rice blast fungus (Chanclud et al. 2016) and to differing levels of virulence among *U. maydis* strains (Morrison et al. 2017). Further investigation of the host CK response regulator genes may help to unveil new approaches leading to better understanding of susceptibility and resistance factors in both cedar and apple trees affected by *G. juniper-virginianae* (Arya et al. 2014; Sekhwal et al. 2015).

Absciscic acid was present in a great abundance in all tissues tested; however, ABA patterns were different between the two host plant species when comparing infected tissue to the controls. Sporulation galls contained ABA levels that were, astonishingly, 28-fold greater than the already high levels of healthy cedar branchlets. Such high ABA concentration may be

attributed to cedar response to stress factors, namely rust development and sporulation occurring in the galls. However, high levels of ABA were detected in both wet and dry telial horns, which are composed solely of rust teliospores; thus, we suggest that at least part of the ABA was of fungal origin. Production of significant quantities of ABA was previously reported in axenic cultures of another fungal pathogen, *U. maydis* (Bruce et al. 2011). Exogenous ABA application on susceptible rice leaf sheaths has been shown to increase susceptibility and disease responses to rice blast fungus, *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (Koga et al. 2004). Based on the available literature and the results of this investigation we propose that the ABA derived from *G. juniper-virginianae* can mediate host susceptibility within the cedar system. Extraordinarily high levels of ABA detected in the sporulation galls may be attributed to their extensive tissue proliferation. Cedar galls are large morphological structures that undergo rapid and recurrent changes of moisture content required for teliospore production and germination. This part of rust life cycle involves precise osmotic regulation and ABA is known as an important regulator of osmotic pressure and water permeability through aquaporins (Beaudette et al. 2007) and thus, may assist in promoting a moisture regulated environment for the benefit of the pathogen (Cao et al. 2011).

Contrary to the pattern in cedar, ABA level was higher in the control within the crabapple system, for which healthy leaves contained more ABA than in the leaves of the infected trees. This is not consistent with the hypothesis that it is involved with a stress response mechanism induced by the fungus. However, we consider the option that each host plant species responds uniquely to the pathogen presence and in the stage of apple leaf rust ABA may not be required

and therefore not produced by the pathogen but instead, may be derived solely from the stressed apple plant. The different mechanism of ABA may be a reflection of the different manifestations of the disease whereby the cedar responds with a large gall structure and the apple tree has a less complex fungal infection spot.

Conclusion

The observed dynamics in cytokinin and abscisic acid profiles during cedar-apple rust infection process provides an interesting model to evaluate phytohormone involvement in *Gymnosporangium juniper-virginianae* pathogenesis. Significant changes in hormone composition during fungus lifecycle depict an active relationship between the rust and host plants throughout cedar gall growth and sporulation, and apple rust infection. An abundance of cZ type CKs within the telial horns and the fungus infected tissues points towards their common origin and suggests that fungal derived CKs are a necessary factor for the disease development.

Additional research involving cedar and apple defense response phytohormones might further shed more light onto the parasitic relationship between rust and its hosts leading to better understanding of *G. juniper-virginianae* mode of action in the infected plants and ultimately better strategies involving hormonal treatments in order to deal with infected orchards.

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Table 1. Endogenous hormones and labeled CK standards, scanned for by liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-(ESI)-MS/MS). Deuterated internal standards purchased from OlChemim Ltd. (Olomouc, Czech Republic) (cytokinin) and National Research Council-Plant Biotechnology Institute (NRC-PBI, Saskatoon, Saskatchewan, CA) (ABA) were used for the analysis.

Hormone	² H-labeled internal standard
Nucleotides (NT)	
1. <i>Trans</i> -zeatin riboside-5'-monophosphate (<i>t</i> ZNT)	² H ₅ [9RMP]Z
2. <i>Cis</i> -zeatin riboside-5'-monophosphate (<i>c</i> ZNT)	
3. Dihydrozeatin riboside -5'-monophosphate (DHZNT)	² H ₃ [9RMP]DHZ
4. N ⁶ -isopentyladenosine-5' monophosphate (iPNT)	² H ₆ [9RMP]iP
Ribosides (RB)	
5. <i>Trans</i> -zeatin riboside (<i>t</i> ZR)	² H ₅ [9R]Z
6. <i>Cis</i> -zeatin riboside (<i>c</i> ZR)	
7. Dihydrozeatin riboside (DHZR)	² H ₃ [9R]DHZ
8. N ⁶ -isopentyladenosine (iPR)	² H ₆ [9R]iP
Free bases (FB)	
9. <i>Trans</i> -zeatin (<i>t</i> Z)	
10. <i>Cis</i> -zeatin (<i>c</i> Z)	² H ₃ DHZ
11. Dihydrozeatin (DHZ)	
12. N ⁶ -isopentyladenine (iP)	² H ₆ iP
Glucosides (GLUC)	
13. <i>Trans</i> -zeatin-O-glucoside (<i>t</i> ZOG)	² H ₅ ZOG
14. <i>Cis</i> -zeatin-O-glucoside (<i>c</i> ZOG)	
15. Dihydrozeatin-O-glucoside (DHZOG)	² H ₇ DHZOG
16. <i>Trans</i> -zeatin-O-glucoside riboside (<i>t</i> ZROG)	² H ₅ ZROG

17.	<i>Cis</i> -zeatin-O-glucoside riboside <i>c</i> ZROG	
18.	Dihydrozeatin-O-glucoside riboside (DHZROG)	$^2\text{H}_7\text{DHZROG}$
19.	<i>Trans</i> -zeatin-9-glucoside (<i>t</i> Z9G)	
20.	<i>Cis</i> -zeatin-9-glucoside (<i>c</i> Z9G)	$^2\text{H}_5\text{Z9G}$
21.	Dihydrozeatin-9-glucoside (DHZ9G)	$^2\text{H}_3\text{DHZ9G}$
22.	N6-Isopentenyladenine-7-glucoside (iP7G)	$^2\text{H}_5\text{iP7G}$
23	N6-Isopentenyladenine-9-glucoside (iP9G)	
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Methylthiols (MET)		
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24.	2-Methylthio- <i>trans</i> -zeatin (2MeSZ)	$^2\text{H}_5\text{MeSZ}$
25.	2- Methylthio- <i>trans</i> -zeatin riboside (2MeSZR)	$^2\text{H}_5\text{MeSZR}$
26.	2-Methylthio-N ⁶ -isopentyladenine (2MeSiP)	$^2\text{H}_6\text{MeSiP}$
27.	2-Methylthio-N ⁶ -isopentyladenosine (2MeSiPA)	$^2\text{H}_6\text{MeSiPR}$
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Aromatic cytokinins		
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26	Benzyloaminopurine (BA)	$^2\text{H}_7\text{BA}$
27	Benzyloaminopurine riboside (BAR)	$^2\text{H}_7\text{BAR}$
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28	Absciscic acid (ABA)	$^2\text{H}_4\text{ABA}$
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Table 2. Concentrations of CKs and ABA isolated from cedar branchlet control, pre-sporulation galls, sporulation galls, post-sporulation galls, wet telial horns, dry telial horns, apple leaf control and infected leaf (means $\pm$ SE, $n=3$).

Hormone level [pmol g fwt ⁻¹]								
Eastern Red Cedar Host							Crabapple Host	
	Branchlet Control	Pre-Sporulation Gall	Sporulation Gall	Post Sporulation Gall	Wet Telial Horn	Dry Telial Horn	Leaf Control	Infected Leaf
cisZNT	-	-	20.65 $\pm$ 3.14	12.89 $\pm$ 3.51	3.53 $\pm$ 0.37	-	28.20 $\pm$ 15.64	481.33 $\pm$ 17.41
iPNT	5.37 $\pm$ 0.64	3.53 $\pm$ 0.14	4.29 $\pm$ 0.19	5.17 $\pm$ 0.38	-	-	37.16 $\pm$ 4.63	69.58 $\pm$ 4.19
Total	5.37$\pm$0.64	3.53$\pm$0.14	24.93$\pm$3.12	18.06$\pm$3.83	3.53$\pm$0.37	-	65.36$\pm$20.04	550.91$\pm$21.25
DZR	3.33 $\pm$ 0.68	5.11 $\pm$ 0.03	-	4.34 $\pm$ 0.20	-	-	-	-
tZR	1.74 $\pm$ 0.20	1.30 $\pm$ 0.25	3.47 $\pm$ 0.63	3.57 $\pm$ 0.68	-	-	-	-
cisZR	-	1.25 $\pm$ 0.16	-	2.18 $\pm$ 0.34	1.24 $\pm$ 0.11	-	9.57 $\pm$ 1.52	160.30 $\pm$ 3.25
iPR	20.24 $\pm$ 1.72	5.75 $\pm$ 1.10	31.31 $\pm$ 3.49	7.28 $\pm$ 0.70	-	-	327.07 $\pm$ 37.68	200.76 $\pm$ 10.85
Total	25.31$\pm$1.99	13.41$\pm$0.88	34.78$\pm$3.78	17.37$\pm$1.58	1.24$\pm$0.11	-	336.64$\pm$39.03	361.06$\pm$8.31
DZ	-	-	-	-	-	-	-	29.73 $\pm$ 0.89
tZ	-	-	19.26 $\pm$ 2.60	-	-	-	4.07 $\pm$ 0.48	14.63 $\pm$ 1.13
cisZ	-	-	26.84 $\pm$ 3.95	-	-	-	-	99.63 $\pm$ 11.67
iP	-	-	9.51 $\pm$ 1.04	-	-	-	8.50 $\pm$ 0.65	7.06 $\pm$ 0.35
Total	-	-	55.61$\pm$1.14	-	-	-	12.57$\pm$0.66	151.04$\pm$11.43
tZROG	-	-	-	-	-	-	24.47 $\pm$ 4.22	32.10 $\pm$ 3.07
tZOG	-	2.71 $\pm$ 0.43	-	7.92 $\pm$ 1.73	-	-	9.71 $\pm$ 1.23	9.03 $\pm$ 1.16
Total	-	2.71$\pm$0.43	-	7.92$\pm$1.73	-	-	34.18$\pm$3.75	41.13$\pm$4.01
MeSZR	-	-	-	-	-	-	29.12 $\pm$ 1.38	90.21 $\pm$ 6.47
Total	-	-	-	-	-	-	29.12$\pm$1.38	90.21$\pm$6.47
ABA	96740.45 $\pm$ 204414.48	870360.38 $\pm$ 5679.11	2717446.85 $\pm$ 205476.73	1967788.68 $\pm$ 204414.48	30170.55 $\pm$ 8094.12	41515.73 $\pm$ 2877.68	124831.41 $\pm$ 8327.92	82532.04 $\pm$ 8870.5

Figure captions

Figure 1. *Gymnosporangium juniper-virginianae* life cycle: (C0) red cedar branchlet; (C1) pre-sporulation gall; (C2) sporulation gall with wet telial horns containing teliospores; (C3) teliospores isolated from telial horns; (C4) basidia produced from germinating teliospores; (C5) basidiospores discharged into the air; (C6) sporulation gall with dry telial horns exposed; (C7) post-sporulation gall after release of teliospores; (A0) apple tree leaf; (A1) pycnia appearing on top of apple leaf; (A2) aecium formed on the lower side of apple leaf; (A3) aeciospores after being ejected from the aecium (more details in the text).

Figure 2. Levels of total cytokinins in the analyzed cedar, rust and apple tree samples (means $\pm$ SE, $n=3$).

Figure 3. Total cZ type cytokinin concentrations including FB, RB, and NT in the analyzed: A) cedar and rust, and B) apple tree samples (means $\pm$ SE, $n=3$).

Figure 1. *Gymnosporangium juniper-virginianae* life cycle: (C0) red cedar branchlet; (C1) pre-sporulation gall; (C2) sporulation gall with wet telial horns containing teliospores; (C3) teliospores isolated from telial horns; (C4) basidia produced from germinating teliospores; (C5) basidiospores discharged into the air; (C6) sporulation gall with dry telial horns exposed; (C7) post-sporulation gall after release of teliospores; (A0) apple tree leaf; (A1) pycnia appearing on top of apple leaf; (A2) aecium formed on the lower side of apple leaf; (A3) aeciospores after being ejected from the aecium (more details in the text).

144x114mm (300 x 300 DPI)

Figure 2. Levels of total cytokinins in the analyzed cedar, rust and apple tree samples (means $\pm$ SE, n=3).

147x88mm (300 x 300 DPI)

Figure 3. Total cZ type cytokinin concentrations including FB, RB, and NT in the analyzed: A) cedar and rust, and B) apple tree samples (means $\pm$ SE, n=3).

106x48mm (300 x 300 DPI)