Cytokinins in nematodes: the potential role of cytokinins in soybean (*Glycine max*) resistance to soybean cyst nematode (*Heterodera glycines*)

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Abstract

Cytokinins in nematodes: the potential role of cytokinins in soybean (*Glycine max*) resistance to soybean cyst nematode (*Heterodera glycines*)

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To investigate cytokinins (CKs) in nematodes, CK profiles of a free-living *Caenorhabditis elegans* and a plant parasitic *Heterodera glycines* (soybean cyst nematode, SCN) were determined at the egg and larval stages. SCN had higher total CK level than *C. elegans*; however, CKs in SCN were mostly inactive precursors, whereas *C. elegans* had more bioactive forms. This is the first study to show that methylthiols are present in nematodes and may affect plant infection. In infectious SCN larvae, methylthiol levels were much higher than in eggs or *C. elegans* larvae. Furthermore, The CK profiles of SCN-susceptible and resistant *Glycine max* cultivars at three developmental stages revealed that, regardless of the resistance level, SCN infection caused an increase in root CKs. One resistant cultivar, Pion 93Y05, showed significantly high levels of bioactive N^6-isopentenyladenine (iP) in the non-infected roots which indicated a potential role of CKs in soybean resistance to SCN.

Keywords: Nematode, Soybean, Cytokinins, HPLC-MS/MS, SCN resistance.

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Abbreviations

AAFC = Agriculture and Agri Food Canada

ABA = Abscisic acid

CK = Cytokinin

FBCKs = Free bases (DHZ + tZ + cZ + iP)

RBCKs = Ribosides (DHZR + tZR + cZR + iPR)

NTCKs = Nucleotides (DHZNT + tZNT + cZNT + iPNT)

cZ = *cis*-zeatin

cZR = *cis*-zeatin 9-riboside

cZNT= *cis*-zeatin nucleotide

DHZ = Dihydrozeatin

DHZR = Dihydrozeatin 9-riboside

dNTPs = Deoxynucleotide triphosphates

DAS = Days after sowing

DWt = Dry weight

DMAPP = Dimethylallyl pyrophosphate

GLUC = Glucosides (DZOG + tZOG + cZOG + DZROG + tZROG + cZROG + DZ9G + tZ9G + cZ9G)

HESI = Heated electrospray ionization

HMBDP = Hydroxy- methylbutenyl diphosphate

IPT = Isopentenyltransferase

iP = N^6-isopentenyladenine

iPR = N^6-isopentenyladenine 9-riboside

iPNT= N^6-isopentenyladenine nucleotide

LB broth = Luria-Bertani broth

LC-MS/MS = Liquid chromatography - tandem mass spectrometry

LOG = "Lonely Guy" cytokinin-activating enzyme

2MeSCKs = Methylthiols (2MeSZ + 2MeSiP + 2MeSZR + 2MeSiPA)

2MeSiP = 2Methylthiol-isopentenyladenine

2MeSiPR = 2Methylthiol-isopentenyladenosine

2MeSZ = 2Methylthiol-zeatin

2MeSZR = 2Methylthiol-zeatin riboside

MVA = Mevalonate pathway

MEP = Methylerythritol phosphate pathway

m/z = Mass to charge ratio

HPLC = High performance liquid chromatography

NGM = Nematode growth medium

QTL = Quantitative trait loci

RH = Relative humidity

R1 = Reproductive stage 1

RPM = Revolution per minutes

SCN = Soybean cyst nematode

tZ = *trans*-zeatin

tZR = *trans*-zeatin 9-riboside

tZNT= *trans*-zeatin nucleotide

TBE buffer = Tris/Borate/EDTA buffer

VE = Vegetative emergence

V1 = Vegetative stage 1

1 Introduction

1.1 General introduction

Phytohormones are a group of naturally occurring, organic signal molecules which influence plant physiological processes at very low concentrations ($<10^{-8}$ M; Hoyerová et al., 2006). In 1881, Charles Darwin first documented the existence of a hormone in plants that was later named as auxin (Whippo & Hangarter, 2006). The term 'phytohormone' was coined by Went and Thimann in 1937 (Thimann & Went, 1937). The initial research identified five major classes of phytohormones: auxin, cytokinins, gibberellins, ethylene and abscisic acid (Rost & Weier, 1979). This list was later expanded to include brassinosteroids, jasmonates, salicylic acid and strigolactones which are now considered as major plant hormones. These signaling molecules are involved in various aspects of plant growth and development including seed dormancy and germination, cell division and differentiation, root and shoot formation, apical dominance and fruit setting (Thimann & Went, 1937; Davies, 2010; Kieber & Schaller, 2014; Murai, 2014). They also play critical roles in regulating plant stress tolerance to promote survival and acclimation to varying environments (Fahad et al., 2015). While some phytohormones, such as jasmonic acid and salicylic acid, are often associated with plant response to pathogen attack, increasing attention has been recently placed on cytokinins (CKs) and their role in plant-pathogen interactions, as they have been identified as one of the key virulence factors in plant pathogenesis (Spallek et al., 2018). Although phytohormones are commonly associated with plants, they are also present in a wide variety of organisms including nematodes (Dimalla & Van Staden, 1977; Bird & Loveys, 1980; De Meutter et al., 2003; Siddique et al., 2015). However, little is known about the role and importance of phytohormones in nematodes. Therefore, in this thesis, the role of CKs in nematodes will be investigated in two ecologically different species of nematodes; soil free-living; *Caenorhabditis elegans* and the plant parasitic nematode; *Heterodera glycines* Ichinohe (soybean cyst nematode, SCN), at different developmental stages to understand the complex picture of CKs involvement in nematode physiology. In the second part of the thesis, the changes of CKs profiles during plant-nematode interactions will be examined in four soybean cultivars that differ in their SCN resistance level, in response to SCN infection.

1.2 Nematodes

Nematodes are extremely abundant and diverse animals. They are unsegmented, mostly microscopic roundworms belonging to phylum Nematoda (Garcia, 1999). Approximately 40,000 species have been identified and this includes free-living and parasitic nematodes. It has been found that 40% of the described species of nematodes are free-living, feeding on bacteria, fungi and protozoans (Lambert & Bekal, 2002). These nematodes, although not widely studied, are generally beneficial to the decomposition of organic matter in soil, where they, along with other microorganisms such as bacteria and fungi, aid in the recycling of nutrients. They are also sensitive to changes in the ecosystem (Neher et al., 1998). Approximately, 45% of all known nematode species are parasitic to animals and 15% are plant parasites. Because of their importance to agriculture, plant-parasitic nematodes (PPNs) are extensively studied in nematology. Estimated yield losses caused by PPNs are around 12.3% ($157 billion dollars) per year worldwide (Singh et al., 2015). Out of over 4000 identified species of PPN, only a few genera, for example, *Meloidogyne* and *Heterodera,* possess a great economic threat to agriculture*.* Collectively, they cause an estimated $80–$118 billion dollars worldwide in yield losses annually (Decraemer and Hunt., 2006; Nicol et al., 2011; Bernard et al., 2017).

As obligate biotrophs, adult plant parasitic nematodes such as those from *Heterodera* or *Meloidogyne* genera are entirely dependent on plant-derived nutrients to fulfil their energy requirements throughout their life cycles. The involvement of various plant hormones in the plant–nematode interaction has been identified and it was recently found that CKs and gibberellins are important for nematode feeding site formation in their host plants. Several plant pathogens including nematodes are known to produce plant hormones such as auxin, gibberellins, abscisic acid, and cytokinins, which contribute to their virulence and thus, may be classified as virulence factors (Goverse & Bird, 2011; Chanclud et al., 2016). In general, the role of phytohormones in nematode physiology is poorly defined, although recent analyses shed more light on the role of nematode derived CKs in the *Heterodera schachtii*-Arabidopsis interaction (Siddique et al., 2015). Clearly, more information on different systems is necessary as each plant-nematode interaction is affected by the ability of the plant to respond to signals produced or triggered by the pathogen. Therefore, in this thesis, *Caenorhabditis elegans,* and *H. glycines,* will be studied as the representatives of free-living nematodes and PPN, respectively, to determine the physiological role of CKs in nematodes. In the following sections (1.2.1 and 1.2.2), *C. elegans* and *H. glycines* will be discussed to provide background on why these species were selected.

1.2.1 Free-living nematodes

***Caenorhabditis elegans* (*C. elegans*)**

The free-living nematode, *C. elegans* has been used as a model organism in molecular genetics and developmental biology since 1970 because of: its small size, large brood size, ease of cultivation, low maintenance expense, long-term cryopreservation, small genome, quick reproduction, few cell types and self-fertilization (Brenner, 1974; Corsi et al., 2015; Fire et al., 1998). *C. elegans* is a bacteriovore, free-living soil nematode species found worldwide. An adult is about 1mm long and newly hatched larvae are 0.25 mm long (Corsi et al., 2015; Wood, 1988). It was the first multicellular organism to have its whole genome sequenced (*C. elegans* sequencing consortium, 1998) and, as of 2012, remains the only organism to have its connectome (neuronal "wiring diagram") completed (Sarma et al., 2018). Many of the genes in the *C. elegans* genome have functional counterparts in humans, which makes it a useful model in biogerontology (Kadlecová et al., 2018), neurobiology (Corsi et al., 2015) as well as to study of human diseases including congenital heart disease and kidney diseases (Culetto, 2000).

Life cycle

C. elegans has a short life cycle that goes through an embryonic stage and four larval stages before reaching adulthood. It exists mostly as a self-fertilizing hermaphrodite (figure 1). Males arise at a frequency of <0.2% (Corsi et al., 2015). The cycle completion can take 2 to 4 days depending on the temperature (optimum development at 20^{O}C). The larval stage L1 is 12-16 hr long and the other larval stages are 7-12 hr long (Sulston and Hodgkin, 1988; Corsi et al., 2015). The body size of the worm increases throughout the four larval stages with several characteristic traits of growth and development present at each stage (Porta-de-la-Riva et al., 2012).

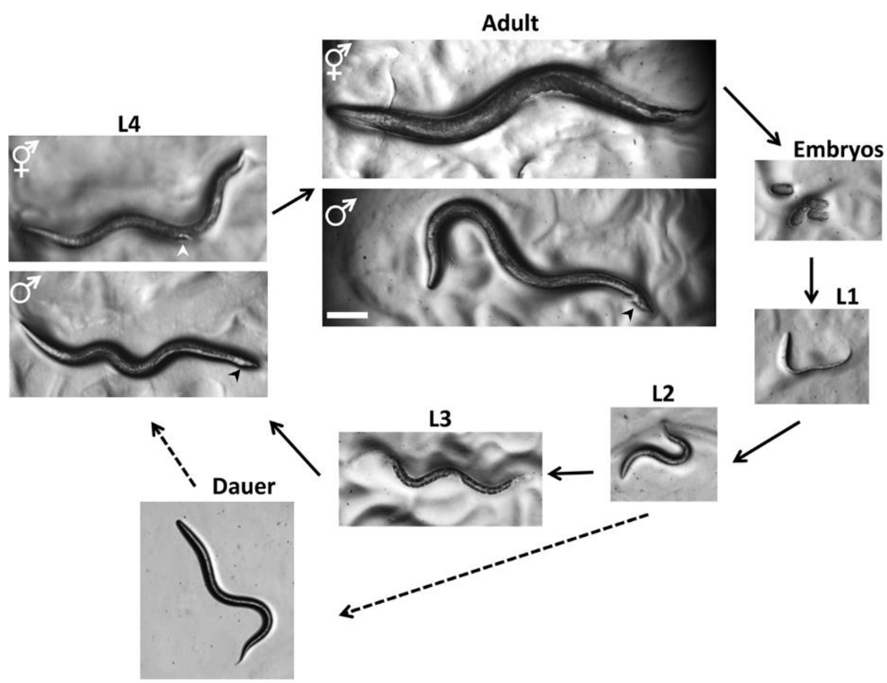


Figure 1 - Life cycle of C. elegans on NGM agar plates in presence of bacterial lawns except in the dauer (a dormant phase). L= larva. Scale bar 100 µm. (Corsi et al., 2015)

The sex of individuals is not easily distinguished until the L4 stage. The two sexes, hermaphrodite and male, can be distinguished by the wider diameter and tapered tail of the hermaphrodite and slimmer width and fan-shaped tail of the male (figure 1, black arrowhead). An inactive period at the end of each stage called lethargus

(Raizen et al., 2008; Trojanowski & Raizen, 2016) ends with the molting of the old cuticle. When foods are depleted in response to overpopulation, the L2 larvae molt into dauer larvae which can survive for several months (Stiernagle, 2006). The dauer larvae are thinner than all the other larval stages (figure 1, dauer). Hermaphrodites live several more weeks before dying after the reproductive stage. The role of CKs in *C. elegans*, a non-parasitic, soil free-living nematode, has not been investigated. The majority of CK research involving nematodes has been associated with plant parasites because of their agricultural importance. Thus, in this study, the model organism *C. elegans* was considered as the ideal species to evaluate role of endogenous CKs in free-living nematode.

1.2.2 Plant parasitic nematodes

In nematology, much attention has been placed on plant parasitic nematodes (PPN) because of their agricultural impact. The first account of plant parasitic nematodes was documented by Needham in 1743 in wheat seeds (Lambert & Bekal, 2002). In 1859 Schacht first reported a nematode disease on sugar beet in Germany, followed by Schmidt in 1871 who described the cyst forming sugar beet nematode *Heterodera schachtii*. Based on the nutrient ingestion, below ground (soil) PPNs are classified into three types; ectoparasitic, semi-endoparasitic and endoparasitic. Each type has both migratory and sedentary species. Ectoparasitic nematodes, such as *Xiphinema americanum* (dagger nematode) travel among different hosts for nutrient intake and are known to transmit diseases from one plant to another (MacFarlane et al., 2016). Semi-endoparasitic species such as reniform nematode (*Rotylenchulus reniformis)* hatches from the egg as juvenile 2

(J2), then quickly molts in the soil to the adult stage without feeding (Lambert & Bekal, 2002). The anterior end of an adult female enters into the suitable host root and forms a permanent feeding cell (Lambert & Bekal, 2002). The J2 of endoparasitic nematodes invade the plant near the tip of a root and migrate through the tissue to the developing vascular cells to establish a permanent feeding site. Once the feeding site is formed, the juvenile nematode rapidly becomes sedentary, feeds, enlarges and molts three times before reaching the adult stage. Adult females enlarge considerably into a sack-like shape and are capable of laying large numbers of eggs. The eggs of endoparasitic nematodes are retained inside the hardened dead female body, called a cyst (Shanks et al., 2016). Of all the important plant-parasitic nematodes, cyst forming species such as *Heterodera sp.* (cyst nematode) and *Meloidogyne sp.* (root-knot nematode) posse the greatest risk in agriculture because the cysts have the ability to persist for a long period of time in the field. The eggs in the cyst are in a dormant state and they hatch slowly over years, usually in response to signals from host roots.

Plant hormones play an important role in nematode feeding site formation. Previous studies identified distinct expression of CK biosynthesis, catabolism and signaling genes in Arabidopsis in response to infection by *Heterodera schachtii* (beet cyst nematode; BCN) and *Meloidogyne incognita* (root-knot nematode; RKN) (Williamson & Gleason, 2003; Dowd et al., 2017; Siddique & Grundler, 2018). The existing studies have concentrated primarily on the model system *Arabidopsis-Heterodera schachtii*; however, each plant-nematode interaction is unique in terms of the ability of the plant to respond to signals produced or triggered by the pathogen. Therefore, in the present study, I will investigate the role of CKs in *H. glycines* which is currently the number one threat to soybean production in North America.

***Heterodera glycines* (soybean cyst nematode)**

Heterodera glycines Ichinohe (Soybean cyst nematode; SCN;) is an obligate, sedentary endoparasitic nematode of soybean (*Glycine max* (L.) Merr.) (Niblack et al., 2009). It has been ranked as the most damaging pathogen in the United States and Canada for more than 60 years following its first discovery (Allen et al. 2017). It was first discovered in northeast China in 1899 (Li et al., 2011) and believed to be introduced to North America from Japan in 1954 (North Carolina, USA) (Riggs, 1977; Winstead et al. 1955). SCN was first noticed in Kent County, Ontario, Canada in 1987 and was reported in Quebec in 2014 (Mimee et al., 2014). Approximately 7-10% of soybean yield loss occurs because of SCN infection worldwide annually. It suppresses seed yield more than any other single soybean pathogen (Wrather & Koenning, 2006, Tylka & Marett, 2014).

Life cycle

SCN has six life stages: the egg, four larva/juvenile stages (J1-J4), and the adult stage (figure 2). The life cycle can be completed in 24 to 30 days under optimum environmental conditions (soil temperature 24^{O}C). After embryonic development within the egg to the first-stage juvenile, the nematode goes through four molts to the adult stage. The first molt occurs within the eggshell, and it is the juvenile two (J2) that emerges from the egg. The J2 is the only life stage of SCN that penetrates roots and moves intracellularly to the root vascular tissue using their stylet (short, dark spear with basal knobs inside the tip of the head) to withdraw food and also to secrete protein and metabolites that support the nematode in parasitizing the plant (Lambert & Bekal, 2002; Williamson & Gleason, 2003).

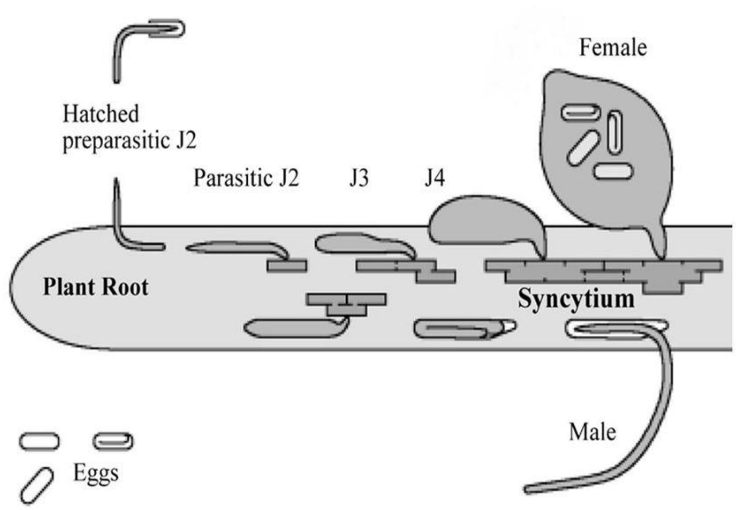


Figure 2- Life cycle of soybean cyst nematode. J = juvenile. (Lilley et al., 2005)

Upon reaching the vascular bundles, the J2's start developing their special feeding site, called a syncytium, which is a large formation of metabolically active cells which becomes multinucleate as neighboring cells are combined by cell wall dissolution and cell fusion (Lambert & Bekal, 2002). These nematode-induced feeding structures function as a conduit between the nematode and plant (Hogenhout et al., 2012). It is the only source of nutrients for nematodes throughout their life and once a J2 starts feeding it becomes immobile. A dramatic change occurs in the morphology and physiology of the host while the nematode undergoes molting towards maturity within the host.

Sex determination of SCN, distinguishable only in the adults, depends on the level of juvenile nourishment. It has been documented that a higher percentage of males is produced when the nematodes are under stress, such as when they occupy a dying host (Lambert & Bekal, 2002). The adult female SCN is lemon-shaped, ranging from 500 to 900 µm long and 200 to 700 µm wide (figure 3). They swell and molt through the juvenile life stages until they protrude from the plant roots. Male nematodes are 1,200 to 1,400 µm long and 26 to 30 µm wide (figure 4), and not as swollen as adult females. They migrate out of the root into the soil and fertilize the females. The males then die or migrate to another host, while the females remain attached to the root system and continue to feed. After fertilization, the females start laying eggs, initially in a mass or egg sac outside the body, then later within the body cavity. Eventually, the entire body cavity of the adult female is filled with eggs, and the female nematode dies. It is the egg-filled body of the dead female that is referred to as the cyst (figure 3). Cysts eventually dislodge and are free in the soil. The walls of the cyst become very tough, providing excellent protection for the 200 to 400 eggs that are enclosed. SCN eggs can survive within the cyst until the proper hatching conditions of the next soybean growing season, or even for several years. It is suspected that exudates from soybean roots provide the hatching signals for the SCN eggs. It has been suspected for a long time that CKs facilitate host invasion by SCN.

Recent studies on *Arabidopsis-H. schachtii* interaction suggest that CKs play the crucial role in nematode feeding site establishment in the root (Siddique et al., 2015; Dowd et al., 2017). The same studies reported that virulence of *H. schachtii* and *M. incognita* is weaker in CK-deficient Arabidopsis mutant plant (*CKXs* overexpressing or *ahk3,4* double mutant) which supports a host plant CK requirement for optimal parasitism. Thus, based on the reports on *H. schachtii* and *M. incognita*, CKs are considered as one of the key virulence factor in nematode parasitism. The role of CKs in SCN has never been studied in previous research or compared with other species. Since the virulence of plant parasitic nematodes is known to be host-specific, in the following section, the mechanistic knowledge of the SCN resistance genes in soybean will be discussed in relation to SCN virulence.

Figure 3- Egg-filled females (whitish) and cysts (tan and brown) of SCN. (source: Greg Tylka, Iowa State University, https://crops.extension.iastate.edu/how-interpret-scn-soil-test-results)

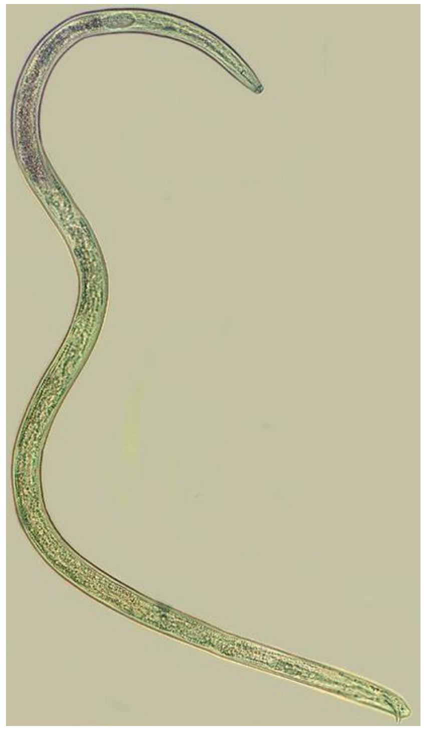


Figure 4-Light micrograph of an adult male soybean cyst nematode. (source: Jonathan D. Eisenback, Virginia Polytechnic Institute and State University, Bugwood.org.https://www.forestryimages.org/browse/detail.cfm?imgnum=5442354)

SCN virulence and host resistance

Variability in nematode virulence is determined by the ability of SCN populations to reproduce on resistant soybean cultivars. This variability was recognized soon after SCN was discovered in North Carolina in 1954, since the nematode was able to adapt to, and overcome, resistant soybean cultivars. At that time, the terms "physiological strains" or "biotypes" were used to describe this variability. By the 1970's the term "race" was used. The first concept of race in SCN was introduced in 1970 (Golden et al., 1970) using the following resistant differentials: Pickett, Peking (PI 548402), PI 88788, and PI 90763 and the susceptible check Lee. Four patterns (Races 1, 2, 3, and 4) of SCN population were observed using these differentials and later expanded to 16 races (Riggs, 1987). A new classification scheme was proposed in 2002 and called the HG Type test (HG stands for *H. glycines*) (Niblack et al., 2002). An HG Type is a description or profile of an SCN population based on the nematodes' ability to reproduce on resistant soybean cultivars. There are seven SCN resistant soybean cultivars that are used as indicator (seven genetic sources of SCN resistance) in HG Type test. The resistant cultivars are PI 548402 (Peking), PI 88788, and PI 90763, PI 437654, PI 209332, PI 89772, and PI 548316 (Cloud). Instead of using the susceptible cultivar Lee, the new scheme uses Lee 74 as a susceptible check. The HG-type of SCN population is determined by using a Female Index (FI).

$$\mathrm{FI} = \frac{\text{average number of females on indicator line}}{\text{average number of females on Lee74}} \text{ X 100}$$

The levels of resistance in soybean cultivars are determined based on FI values (Table 1). SCN resistance in soybean is measured by counting the number of females that develop in an indicator cultivar compared to the number that develop on similarly inoculated susceptible cultivar and expressed as the female index (FI) (Matthews & Reham, 2016; Niblack et al., 2009; Tylka & Mark, 2010). Soybean cultivars are generally classified as highly resistant to SCN if the FI is less than 10% (Schmitt and Shanon, 1992).

Table 1: Classification of the soybean cultivars for resistance to the soybean cyst nematode based on the female index (Schmitt & Shannon, 1992).

FI	Rating
< 10	Resistant (R)
10 to < 30	Moderately resistant (MR)
30 to < 60	Moderately susceptible (MS)
60<	Susceptible (S)

The HG type system is useful for making cultivar recommendations for SCN infested fields. It uses a 1-7 numbering scheme and each number corresponds to a specific PI (Table 2). For example, HG 2.5.7 refers to a biotype that reproduces on PI numbers 2, 5 and 7. This scheme is open ended and easily adaptable to different geographic areas.

Table 2: Indicator soybean lines for HG Type classification of genetically diverse populations of *H. glycines* (Tylka, 2016).

Number	Indicator line
1	PI 548402 (Peking)
2	PI 88788
3	PI 90763
4	PI 437654
5	PI 209332
6	PI 89772
7	PI 548316 (Cloud)

To date, there is no resistant cultivar that shows 100% resistance to SCN. Inheritance of resistance to SCN is quantitative and complex. It involves several dominant and recessive genes. Three recessive genes *rhg1, rhg2* and *rhg3* have been reported in the SCN resistant soybean cultivar Peking (Caldwell et al., 1960), while the forth resistance gene *rhg4* was reported by Matson and Williams (1965) in Peking and PI 437654, but not in PI 88788 or PI 209332 (Brucker et al., 2005). A dominant gene, *rhg5* was identified in PI 88788 (Rao-Arelli et al., 1992; Rao-Arelli, 1994). The *rhg1* locus is frequently found in soybean germplasm and has the greatest impact on the SCN resistance (Melito et al., 2010). Peking, PI 88788, PI 437654, PI 209332 and PI 90763 have this locus which provides soybean resistance to many common SCN populations (Concibido et al., 2004). Although no single locus can provide complete resistance to all races of SCN, the combinations of these major loci can result in higher levels of resistance (Concibido et al., 2004). Genetic mapping efforts uncovered numerous locations of SCN resistance quantitative trait loci (QTL) in many PIs. The linkage groups (LGs): A1, A2, B1, B2, C1, C2, D1a, D2, E, F, G, H, I, J, L, M, and N have been associated with SCN resistance where linkage group G has the most QTL regions (four) associated with SCN resistance, followed by LGs B1, C2, and D2 with three, and LGs A1, B2, D1a, E, and M with two; the rest of the LGs have one QTL (Concibido et al., 2004). Among the sources of resistance, Peking has nine, the highest number of QTL associated with resistance, followed by eight in PI 438489, and six in PI 437654 (Concibido, Diers, & Arelli, 2004). Peking' (PI 548402), PI 88788, and PI 437654 were shown to carry resistance loci effective against multiple nematode races and have been used as sources of resistance for commercially grown soybean cultivars (Concibido et al., 1977, 2004). To date, CK involvement in SCN resistance mechanisms has not been reported in soybean. So, in the second part of current study, I will investigate the role of CKs in SCN resistance in different SCN resistant soybean cultivars.

1.3 Cytokinins (CKs)

Cytokinins (CKs) represent a family of plant hormones that primarily promote cell division (Sakakibara, 2006; Kieber & Schaller, 2014) and also affect a number of plant growth and developmental processes, including shoot initiation and growth, leaf senescence, nutrient uptake and embryonic development, as well as the response to biotic and abiotic factors (Mok & Mok, 2001; Kieber, 2002; Quesnelle & Emery, 2007; Kamada- Nobusada & Sakakibara, 2009; Kieber & Schaller, 2014; Morrison et al., 2015). CKs are N^6 substituted adenine derivatives carrying an isoprenoid or aromatic side chain (Sakakibara, 2006; Spíchal, 2012; Kieber & Schaller, 2014). CKs were first discovered in autoclaved herring sperm DNA by Skoog and Miller (Miller et al.,1955,1956). They identified an active modified adenine, 6-furfurylaminopurine which was named kinetin, an aromatic CK (Miller et al., 1956). An isoprenoid CK, zeatin was later identified in immature maize endosperm as the first natural cytokinin in plants (Letham et al.,1973; 1975). Both isoprenoid and aromatic CKs are naturally occurring in plants, however, isoprenoid CKs are more abundant and predominant (Sakakibara, 2006). CK homeostasis is dependent on the balance of biosynthesis, interconversion (between different CK forms), transport and degradation (Miyawaki et al., 2004).

1.3.1 Cytokinin biosynthesis

There are two CK biosynthesis pathways identified; the methylerythritol phosphate (MEP) or *de novo* pathway and the mevalonate (MVA) pathway which is also known as tRNA-degradation pathway (Sakakibara, 2006; Kamada-Nobusada & Sakakibara, 2009) (figure 5). Based on their chemical structure, CKs are classified into three major groups: nucleotide (NTCKs), considered precursor forms; riboside (RBCKs), intermediate and potentially active; and free base (FBCKs), which are the most active forms (Sakakibara, 2006). Each group of CKs is further divided into four types which include N^6 isopentenyladenine (iP), *trans*-Zeatin (tZ), *cis*-Zeatin (cZ) and dihydrozeatin (DHZ) (Kamada-Nobusada & Sakakibara, 2009). Glucosides (Gluc) and methylthiols (2MeSCKS) are two other conjugate types that are also widely present in many organisms. Glucosides are formed through glucosylation of the adenine moiety and are known as storage forms of CKs, helping to maintain CK homeostasis (Hou et al., 2004). CK conjugates, that have sulfur and methyl groups attached, form 2MeSCKs. The functions of 2MeSCKs have yet to be determined, although, they are widely occurring and associated with fungal tumor and gall formation (Choi et al., 2011; Morrison et al., 2015; 2017). The most abundant CK types determine the dominant CK pathway in a biological system (Mok & Mok, 2001; Sakakibara, 2006; Kamada-Nobusada & Sakakibara, 2009). According to the CK biosynthesis pathway proposed by Sakakibara (2006), isopentenyltransferases (IPT) catalyze the first reaction in the formation of isoprenoid CKs. Accordingly, tZ and DHZ are biosynthesized in the MEP pathway (figure: 5) which involves transfer of an isopentenyl side-chain derived from either dimethyallyl pyrophosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP) to an adenosine base using ATP, ADP or AMP. The resulting tri-, di- and monophosphate structures of iP type CKs are designated forms of CKNTs. These iP type CKs are converted to the corresponding tZ types CK by CYP735A. A loss of the double bond of tZRMP forms DZRMP which is NT form of the DZ type of CKs. Inactive nucleotide forms of CKs are dephosphorylated to intermediate RBCKs which contain a ribose attachment at the N^9 position to perform different cellular processes depending on the organism. Since RBCKs are unable to interact with most plant CK receptors, they are considered by some as inactive in plants (Lomin et al., 2015). But due to their abundance in vascular bundles, ribosides are often considered as moderately active long-distance signaling molecules (Agurla et al., 2017). Lastly, FBCKs are the most active CK forms and are formed through hydrolysis of the adenosine base of riboside to adenine. The nucleotides can also be directly catalyzed to active free bases via a conversion enzyme encoded by the lonely guy (LOG) gene (Kamada-Nobusada & Sakakibara, 2009).

The MVA or tRNA degradation pathway is the widely accepted source of cZ type CKs (Sakakibara, 2006). tRNA breakdown is involved in this pathway to create *cis*-prenyl-tRNA, the precursor of *cis*-zeatin nucleotide. The transfer of the isopentenyl sidechain is catalyzed by tRNA-isopentenyltransferase (tRNA-IPT) (Sakakibara, 2006). Since the activity of cZ is not well understood, tRNA degradation has often been overlooked as a major contributor to the overall CK pool (Schäfer et al., 2015). However, in some plant species, such as rice, cZs are the dominant CK types, and, in other organisms including fungi, the tRNA degradation pathway is becoming an area of interest for phytohormone research (Gajdošová et al., 2011; Kudo et al, 2012; Morrison et al., 2017). CK oxidase/dehydrogenase (CKX) is the enzyme that catalyzes the irreversible degradation of CKs through the cleavage of the isoprenoid side chain, however, aromatic CKs are resistant to CKX (Galuszka et al., 2000). CK homeostasis in plant systems is primarily maintained by CKX (Jones & Schreiber, 1997). Interestingly, plants with over expression of *CKX* genes are found less susceptible or fairly resistant to nematode infection, particularly, root-knot and cyst nematode infection (Lohar et al., 2004; Siddique et al., 2015; Dowd et al., 2017).

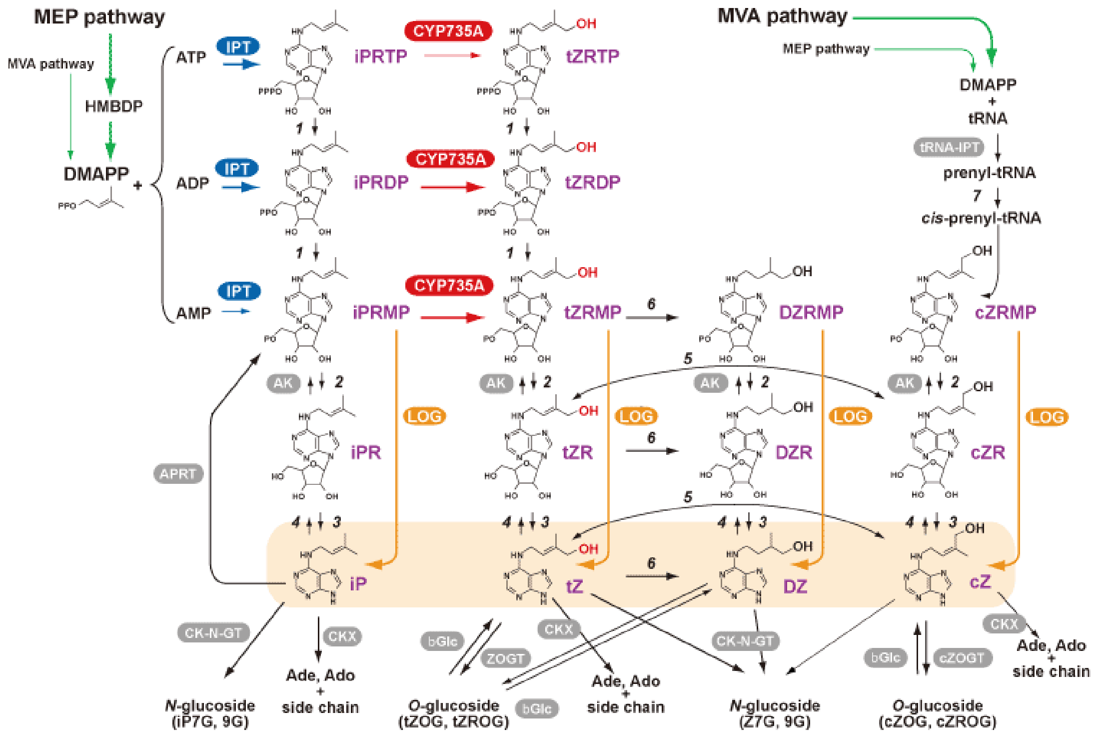


Figure 5-Cytokinin biosynthesis, interconversion and degradation pathway in higher plants (Kamada-Nobusada & Sakakibara, 2009).

1.3.2 Cytokinins in nematodes

Dimalla & Van Staden (1977) first reported the presence of CKs in *Meloidogyne incognita* (root knot nematode; RKN) which, at the time, was believed to originate from plant tissue. Later, it was found that nematodes are able to produce and release CKs (Bird & Loveys, 1980; De Meutter et al., 2003). The presence of a eukaryotic isopentenyltransferase (IPT) acting on tRNA was identified in soil free-living nematode *C. elegans* (Sakakibara & Takei, 2002). Using tRNA-IPT sequence from *C. elegans*, a single gene in *H. schachtii* (*HsIPT*) encoding a tRNA-IPT and one putative full-length and two partial IPT protein sequences were identified in the SCN draft genome (Siddique et al., 2015) providing genetic evidence of the CK biosynthesis pathway in *C. elegans* and SCN. Kadlecová et al. (2018) reported that exogenous application of aromatic CKs (kinetin, meta-topolin and para-topolin) to *C. elegans* prolongs the life span*,* and that kinetin increases thermotolerance and resistance to oxidative stress in *C. elegans in-vitro.* In the same study, endogenous CK profiling confirmed that kinetin does not occur naturally in *C. elegans* although it is effectively absorbed after exogenous exposure and metabolized to kinetin riboside and kinetin riboside-5′-monophosphate. This finding indicates that *C. elegans* has transporter proteins in their plasma membrane that carry CKs into its cells without the need of a CKs receptors. But the identity of any transporters that may link these processes is still unknown.

While nematodes are capable of producing CKs, the role of CKs has never been studied in detail. Therefore, an analysis of CK profiles at different developmental stages of *C. elegans* and *H. glycines* will be conducted for the first time in this thesis research to determine the role of CKs in nematodes. Phytohormones, which are essentially involved in all plant growth and developmental processes, have also been linked to the interaction of nematodes with plants. It is known to play an important role in the development of nematode’s feeding site (syncytium, giant cell) in plant (Williamson & Gleason, 2003; Siddique & Grundler, 2018). Furthermore, nematodes have been shown to produce CKs. These findings indicate that CKs promote the organ sink strength (ability of a sink organ to import assimilates for its growth, development and maintenance) during plant-nematode interaction.

CKs regulate several biological processes (Mok & Mok, 2001; Kieber & Schaller, 2014), that are relevant to the development and function of a syncytium. Changes to endogenous CK levels in plants have been associated with plant pathogen infection (Stirk & van Staden, 2010). In Arabidopsis, CK signaling is activated in response to *H. schachtii* infection. This occurs particularly at the early stage of syncytium development once it is triggered by the nematode and is mediated by two Arabidopsis Histidine Kinase receptors (AHK3 and AHK4) in the host (Siddique et al., 2015; Dowd et al., 2017). CK biosynthesizing genes in *H. schachtii* (*HsIPT* and *HgIPT*) are highly expressed at the early stages of Arabidopsis infection (Elling et al., 2007) suggesting that nematode-secreted CKs might play the vital role in the development of the syncytium. It is assumed that CKs released by plant parasitic nematodes during the infection hijack host CK signaling cues by mimicking plant CK production to increase host susceptibility and promote parasitism (Spallek et al., 2018). Overexpression of *CKXs* in Arabidopsis and use of *ahk3,4* double mutant plants resulted lower susceptibility or moderate resistance to *H. schachtii* and *M. incognita* (Siddique et al., 2015; Dowd et al., 2017) indicating that the host plant CKs are involved in cyst nematode virulence. Additionally, CK signaling is required for optimal parasitism as well as the establishment of the feeding site (Shanks et al., 2016). Although root penetration and syncytium formation by SCN follows similar mechanism in all host plants, however, the syncytium becomes necrotic in resistant cultivars soon after establishment and the starving nematodes die (Kandoth et al., 2011). The progress of this necrotic reaction depends on the source of SCN resistance in plant (Acedo et al., 1984).

To date, CK role in this resistance mechanism has not been assessed in soybean. The potential involvement of CKs in SCN resistance reaction in soybean will be studied in the second part of the thesis.

1.4 Research objectives

Based on the previous studies, CKs are likely to be a key virulence factor for nematode pathogenesis in plants. In *Arabidopsis-H. schachtii* system, *H. schachtii* initially hijack Arabidopsis CK signaling cues by insertion of their own CKs into the host plant that increase the host susceptibility and promote parasitism (Spallek et al., 2018). Additionally, infection assay using *Arabidopsis-H. schachtii* showed that silencing of the *HsIPT* gene in *H. schachtii* caused no change in the size of female nematodes but the number of females and the size of the syncytium were significantly reduced (Siddique et al., 2015) indicating that CKs are produced by *H. schachtii* primarily for the successful parasitism. In contrast, *C. elegans* showed significant physiological change in response to kinetin, a CK type which does not occur naturally in the organism (Kadlecová et al., 2018). However, the role of CKs in nematode physiological processes have not been understood up to date. Therefore, in this thesis eggs and larvae of *C. elegans* and *H. glycines* were selected to conduct a comparative analysis of nematode CK profiles and determine the role of CKs in nematode. In the second part, four soybean cultivars differing in their SCN resistance level were examined for changes in their CK levels due to SCN infection to determine CK involvement in host plant resistance.

1.5 Hypotheses and predictions

The very few research studies conducted on endogenous CKs in nematodes investigated exclusively *M. incognita* or the economically less important *H. schachtii*. SCN has never been investigated to determine if CKs have a role in their infection or pathogenesis. On the other side, CK profiles of *C. elegans* in the previous research were limited to study only selected CKs such as iP or kinetin. This thesis research will be novel in that it will explore the endogenous levels of a broad set of CKs in eggs and larvae of *C. elegans* and *H. glycines* using the advanced techniques of hormone extraction and sample analysis by highly sensitive mass spectrometry to help determine a potential role of CKs in nematodes. As mentioned earlier, CKs are required for nematode feeding site establishment and nematode derived CKs are mainly produced for plant parasitism. In contrast, the role of CKs in *C. elegans* remains elusive, however, they respond to exogenous application of some aromatic CKs which are not naturally produced by the organism.

Therefore, a comparative analysis of CK profiles of these two representatives of free-living and plant parasitic species was applied to determine the role of CKs in nematodes. In the first part of the thesis, it was hypothesized that the CK profiles of *C. elegans* and *H. glycines* would be different. Based on a review of the literature, it was predicted that CK profiles in *H. glycines* would be composed of CKs that are known to be highly biologically active in plants (i.e. free bases) to reflect the purpose of CKs in pathogenesis. More specifically, I predicted that CK profiles of *C. elegans* and *H. glycines* would be different at the larval/juvenile stages of these nematode species as parasitism occurs at this stage.

In the second part of my thesis, a correlation between soybean CK production and SCN resistance was studied. I assumed that SCN affects the CK concentration in soybean roots, regardless of plant level of resistance to SCN. The SCN resistance mechanism in soybean was predicted to be similar to the one existing in CK-deficient *Arabidopsis* plants in response to *H. schachtii* attack. Based on this knowledge, it was hypothesized that the SCN resistance in soybean would be reflected through the different CK dynamics in response to SCN infection. This resistance mechanism will involve the inhibition of soybean CK production upon the SCN infection that, in turn, will prevent syncytium formation.

2 Materials and Methods

2.1 Biological materials

2.1.1 Caenorhabditis elegans

2.1.1.1 Organism description

The wild-type strain (N2) of *Caenorhabditis elegans* was selected as a free-living species of nematode. Stock plates of *C. elegans* were purchased from Carolina Biological Supply Company (Burlington, North Carolina, United States, Item ID#173500). In nature *C. elegans* feeds on soil-dwelling microorganisms but under laboratory conditions they feed on *Escherichia coli* (strain OP50). This strain is uracil auxotroph whose growth is limited on Nematode Growth Medium (NGM). A starter streak plate of *E. coli* OP50 was purchased from the same company as *C. elegans* (Item ID # 155073).

2.1.1.1 Culture media and growth conditions

The protocols for *C.elegans* culture media preparation and growth conditions were adopted and modified from Brenner (1974) and Stiernagle (2006).

Preparation of NGM petri plates

C. elegans was maintained in the laboratory on NGM agar (pH 6.15) plates. To prepare NGM, 3 g NaCl, 17 g agar and 2.5 g peptone were added to 975 mL of deionized water in a 2L Erlenmeyer flask. Sterilization of the media was completed in steam autoclave at 121^{0}C for 30 minutes. Sterilized medium was cooled down in water bath to reach 55^{0}C. 1 ml 1 M $CaCl_2$, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$ and 25 ml 1 M KPO_4 buffer (pH 6.0) were filter sterilized separately and added serially to the media which was swirled to mix. Using sterile technique, the NGM solution was poured into 100x15mm diameter petri dishes (Fisher Scientific International Inc., Pennsylvania, USA) to fill up 2/3 of each plate. The plates were sealed with parafilm and left at room temperature for 2-3 days to allow excess moisture to evaporate and to confirm lack of contaminants.

Preparation of bacterial food source for *C. elegans*

E. coli OP50 was cultured in standard Luria-Bertani (LB) broth. To prepare 1L broth, 10 g Bacto-tryptone (BD Bacto™), 5 g Bacto-yeast (BD Bacto™), and 5 g NaCl were added to 1L of deionized water. Media pH was adjusted to 7 with 1 M NaOH. The media was autoclaved at 121°C for 30 minutes and stored at room temperature until used. To culture *E. coli* OP50, 5 mL of sterile broth was transferred to a sterile test tube aseptically and inoculated with a single bacterial colony from the streak plate. Inoculated culture was allowed to grow overnight at 37°C in a shaker incubator at 250 RPM. The *E. coli* OP50 was ready to seed on NGM plates in the morning. The bacterial streak plates and liquid culture were stored at 4°C for further use.

Bacteria seeding to NGM plate

Using sterile technique, 100µL of *E. coli* OP50 liquid culture was spread onto NGM petri dish to create a large bacterial lawn which helps in visualizing the nematodes. Seeded plates were allowed to grow overnight at room temperature (23°C). These *E. coli* OP50 seeded NGM plates were stored in an air-tight container at room temperature and used within 2-3 weeks.

Nematode culture technique

C. elegans was transferred from the stock plate to bacteria seeded NGM plates by using a "chunking" method every two weeks. A sterile scalpel was used to cut a chunk of agar containing adult nematodes from the stock plate and transferred it to a freshly bacteria seeded NGM plate. The nematodes from the old plate crawled out of the chunk and spread out onto the bacterial lawn of the new plate. Sub-cultured plates were stored in a dark incubator at 21°C for a period of 5 to 7 days. The "chunking" process was sequentially repeated 4 to 5 times to establish healthy and gravid nematode populations. Nematodes were monitored daily by a light microscope at 40× magnification to observe the population growth.

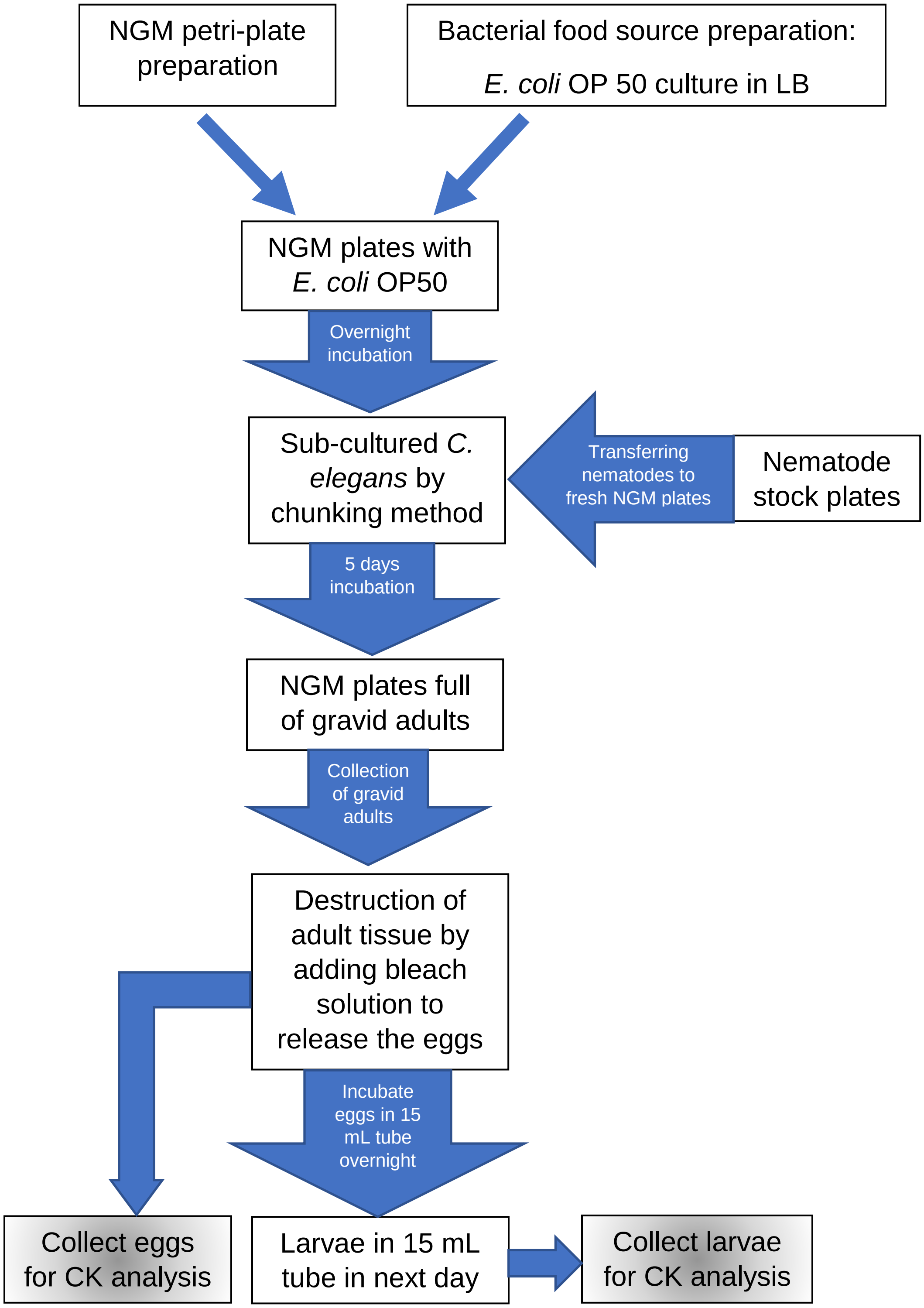


Figure 6-Schematic presentation of *C. elegans in-vitro* culture and eggs and larvae collection for CK analysis.

2.1.1.2 Eggs and larvae isolation

NGM agar plates containing mostly gravid adult *C. elegans* (figure 7) were selected to isolate fresh eggs which was usually 5 days old plates. 5 mL of M9 buffer (pH 6.6-7) (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1mL 1M $MgSO_4$ and water to 1 L and autoclaved) were used to wash each plate under sterile conditions. Washed nematodes from two plate were collected in a 15 mL tube. Collected worms in buffer solution were kept on ice for 15 minutes until they settled down. After 15 min nematodes were centrifuged for 2 min at 400xg and washed with the same buffer solution. This was repeated until the buffer was clear of bacteria. The supernatant was discarded and freshly prepared bleaching solution (H_2O (1.5mL) + NaOH (2.5mL) + NaOCl (1mL) = 5mL) was added to the nematodes and agitated for 5 minutes. Destruction of the adult tissue was monitored using a dissecting microscope. After 5 minutes, the reaction was stopped by filling the tube with M9 buffer. The egg suspension was immediately centrifuged for 1 min at 400xg, the supernatant discarded, and the same washing was repeated three times to remove the bleaching solution. After washing, the egg pellet was resuspended in 1 mL of sterile water and transferred to 2 mL Eppendorf tube (figure 8 A). The egg concentration in each tube was measured using a hemocytometer. Three replicates of egg samples ($1.9X10^5$ to $2.88X10^5$ eggs) were stored in -20^0C and used for CK extraction.

Figure 7-Microscopic view (10x) of adult *C. elegans* in NGM agar plate.

To collect larva, three replicates of a known concentration of egg pellets were incubated with 1 mL of M9 buffer in a 15 mL tube overnight at room temperature with gentle agitation. The next day hatched larvae (figure 8 B) were observed under the microscope.

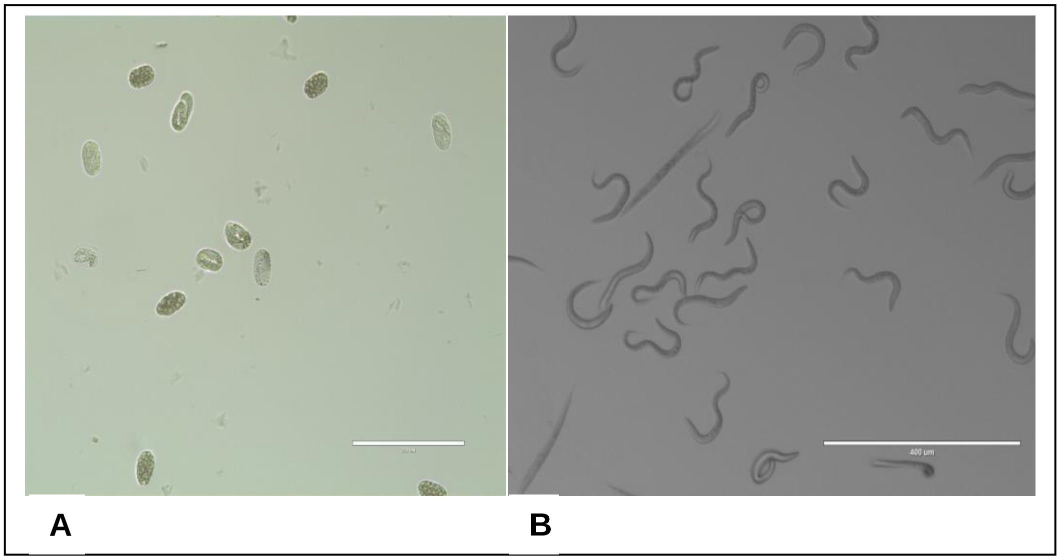


Figure 8- *C. elegans* eggs (A); scale bar 200 µm and larvae (B); scale bar: 400µm

Larvae density determination

The density of larvae was determined by following the *C. elegans* nematode counting protocol (Scanlan et al., 2018). Following the protocol, the larvae were counted and recorded ($1.43X10^5$ to $3.65X10^5$ larvae/replicate). Then the larvae were transferred to 2 mL tube and stored at -20^0C for extraction. The slide was cleaned by spraying with 70% ethanol before and after using.

2.1.2 Heterodera glycines (soybean cyst nematode)

2.1.2.1 Organism descriptions

Soybean cyst nematode (SCN) (race 3 & Hg type 7) was collected from a field near Chatham, Ontario by the lab technician from Harrow Research and Developmental Centre - Agriculture and Agri Food Canada (AAFC) in December 5th, 2017. The removal of cysts from roots and extraction of eggs from cysts were conducted by the same facility staff following the methods modified from Niblack et al. (2009) and Benjamin and Reham (2016). Detailed methods are described in the Appendix (8.2-8.3). The extracted clean eggs suspension was brought to Trent University on December 14th, 2017 in a glass bottle. The egg and larva were isolated for hormone extraction at Trent University.

2.1.2.2 Eggs and juvenile stage isolation

Eggs isolation

The supplied SCN egg suspension in the glass bottle was brought up to 60 mL with sterile water. The bottle was vortexed for a few seconds and 10 mL of the suspension was aliquoted into a sterile 15 mL tube. In total, 6 aliquots were prepared. The eggs suspensions were centrifuged at 400xg for 4 min and the supernatant discarded. Three repeated washes were done with sterile water to remove any exudates. After the final wash, 1 mL of sterile water was added to the tubes and the number of eggs in each tube was counted by using a hemocytometer (figure 9 A) and the egg density was recorded. Three replicates of egg samples ($2.45X10^5$ to $4.29X10^5$ eggs) were transferred to 2mL tubes and stored at -20^0C for CK extraction.

Juvenile isolation

SCN eggs in 15 mL tubes were re-centrifuged and the supernatant discarded. Egg pellets were re-suspended in 1 mL of 4mM $ZnCl_2$ solution which enhances SCN egg hatching and incubated for a week at 50-75 RPM in an agitator for aeration at room temperature. The progression of hatching was monitored using a dissecting microscope. After incubation, the number of living larva (figure 9 B) was counted following the nematode method described by Scanlan et al (2018). The nematodes were washed three times with sterile water in the same tube and transferred to a 2 mL tube. Three replicates of larvae ($5.6X10^4$ to $8.8X10^4$ larvae) were stored at -20^0C for hormone extraction.

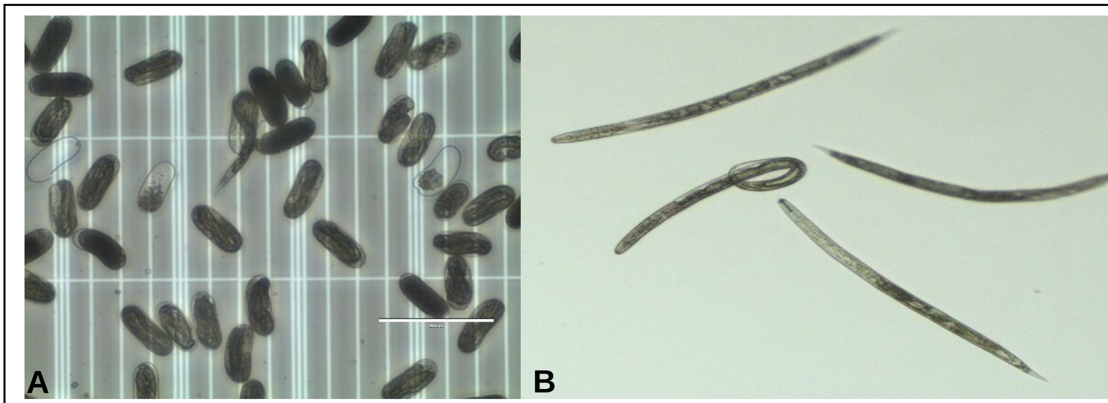


Figure 9- Soybean cyst nematode eggs (A) and freshly hatched juvenile 2 (B). Scale bar: 200 µm

2.1.3 *Glycine max* (soybean)

2.1.3.1 Cultivar description

Four soybean cultivars (Lee74, Essex, P22T69R and P93Y05) representing SCN susceptible and resistant (details in table 3) were selected for this study. The two susceptible cultivars (Lee74 and Essex) were selected to study because they have been used since 1980 as check standards for determining the variety tolerance to SCN in all North American nematology laboratories and the resistant cultivars (P22T69R and P93Y05) are the most popular SCN resistant cultivars among soybean growers in Ontario. The selected SCN resistant cultivars are very similar with respect to agronomic features in SCN infested fields, especially regarding yield performance, although they have different sources of SCN resistance ((Peking (rhg1 and rhg4) and PI88788 (rhg1)) (Concibido et al. 1997).

Table 3: Descriptions of the soybean cultivars used in this study.

Cultivar	SCN susceptibility	Plant introduction (PI)	Maternal Parent X Paternal Parent	Source of SCN resistance	Source/ Breeder
Lee74	Susceptible	PI 548658	Lee 68 X R66-1517	N/A	Soybean Germplasm Collection, USDA
Essex	Susceptible	PI 548667	Lee X S5-7075	N/A	Soybean Germplasm Collection, USDA
P22T69R	Resistant	PI 665321	Pioneer P93M11 X Pioneer P92M21	PI 548402 (Peking)	Pioneer Hi-Bred International, Inc. PVP 201200194
P93Y05	Resistant	PI 659290	Pioneer P92B12 X Pioneer P93M51	PI 88788	Pioneer Hi-Bred International, Inc., PVP 201000119

2.1.3.2 Genotyping for SCN resistance in soybean cultivars

To validate the SCN resistance in the selected soybean cultivars, the presence of *rhg1* gene specific marker was analyzed according to a protocol developed in the Emery Laboratory (Z Zhang; unpublished)**.** The SCN susceptible and resistant soybean cultivars were grown in a greenhouse (Conviron Aurora research greenhouse: heating and cooling temperature setpoints 19°C and 27°C respectively, dehumidification setpoint 80% RH and humidification setpoint 60% RH) in sunshine mix # 1, professional growing mixture (Sun Gro Horticulture, Agawam, Massachusetts, USA) with no added fertilizers. Young leaves were collected from each cultivar at their trifoliate stage. Approximately 22 to 65 ng/µL concentration of DNA was extracted from around 100 mg of ground young leaf tissue using a DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA concentration was measured using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) under 260nm wavelength. All extracted DNA samples were diluted to a concentration of 20 ng/µL using double Nuclease free water. DNA quality was checked on 1% agarose gel in 0.5X TBE buffer.

Genomic DNA template was prepared and normalized for PCR to a DNA concentration of 10 ng/µL for each of the soybean cultivars. Reagents for PCR reactions included:

1) Soybean genomic DNA (10 ng/µL)

2) Ultra-pure distilled molecular grade water (DNase and RNase free)

3) 25mM $MgCl_2$ solution

4) 5X PCR buffer (Promega, GoTaq Flexi DNA polymerase Kit; pH8.5, no magnesium)

5) 10 µM primer pairs (working solutions); (Integrated DNA Technologies, USA)

A. First pair is soybean SCN-resistant *rhg1* gene specific:

Forward primer: Gm-rhg1-F1

Reverse primer: Gm-rhg1-R1

B. Second pair was soybean lectin gene-specific to use as an internal positive control:

Forward primer: Gm-Lec-F2

Reverse primer: Gm-Lec-R2

6) 25mM dNTPs

7) Taq DNA polymerase (5U/μL) (Promega (GoTaq Flexi DNA Polymerase))

Combinations of PCR reaction (volume of 10 μL for each reaction) were prepared on ice following the recipe in the table 4.

Table 4: Recipe for PCR

Components	**Final Volume (μL) for 1 reaction**	**For 25 reactions – Mixture Master Buffer (μL)**
Genomic DNA (10ng/μL)	2.0	
5X PCR buffer	2.0	50.0
25mM $MgCl_2$	2.0	50.0
First pair forward primer (10 μM) - Gm-rhg1-F1	0.2	5.0
First pair reverse primer (10 μM) - Gm-rhg1-R1	0.2	5.0
Second pair forward primer - Gm-Lec-F2	0.2	5.0
Second pair reverse primer - Gm-Lec-R2	0.2	5.0
25mM dNTPs	0.2	5.0
GoTaq Flexi DNA polymerase (5U/μL)	0.1	2.5
Nuclease-Free water	2.9	72.5
Total volume	10.0	250.0

Two μL of genomic DNA was added into each well of PCR plate (96 wells), followed by 8 μL of master mixture. After adding DNA and reaction mixture, the PCR plate was sealed and centrifuged briefly (500 RPM for 30s). The PCR conditions were as follows: 94ºC for 2 min; 35 cycles of 95ºC for 30s, 55ºC for 30s and 72ºC for 30s, and finally 72ºC for 10 min. After PCR amplification of the *rhg1* gene-specific fragment (400 bp) and *lectin* gene-specific fragment (200 bp), 2 μL of 10X loading buffer was added to each reaction. 2% agarose gel electrophoresis was conducted in 0.5X TBE buffer at 120V voltage and run for 45 minutes.

2.1.3.3 Plant cultivation and SCN infection

All the four selected soybean cultivars were grown in the greenhouse and the roots were collected at three developmental stages: vegetative emergence (VE) at 5 days after sowing (DAS), first trifoliate stage or first vegetative stage (V1) at 14 DAS and beginning bloom or first reproductive stage (R1) at 40 DAS (figure 10). The plants at V1 and R1 stage were inoculated with 4000 SCN eggs but at VE stage no SCN was inoculated. This part of the project was conducted at Harrow Research and Developmental Centre – AAFC by their lab technician (George Stasko) and the frozen roots were brought to Trent University on December 14th, 2017. Detailed methods of soybean cultivation in the greenhouse and root collection are described in the Appendix (8.1).

Approximately 0.2g of lateral roots were sampled from each cultivar. The weight of each sample was measured in a labeled pre-weighted 2 mL tube and recorded. The roots were kept and stored at -20ºC for CKs extraction.

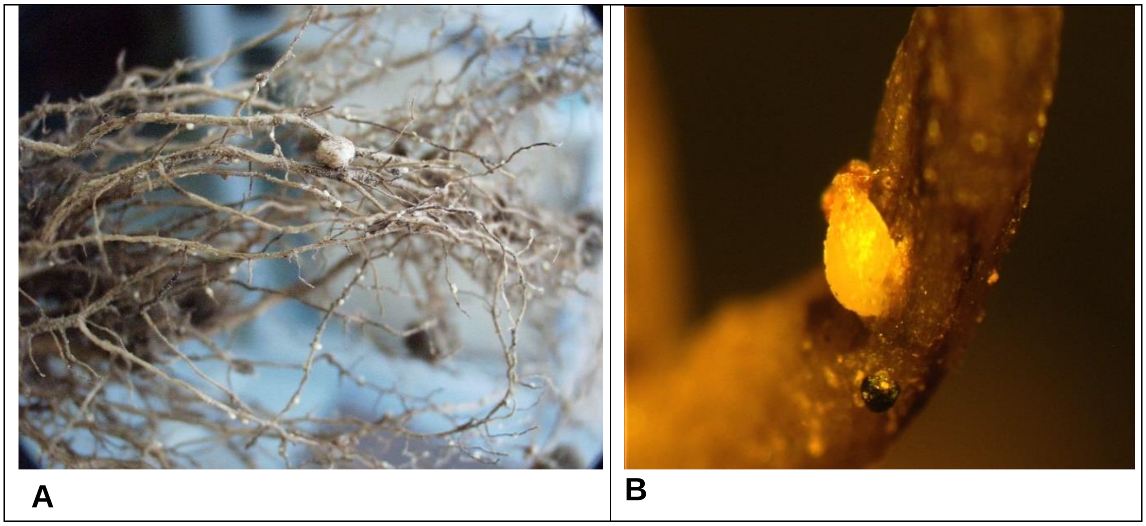


Figure 10-SCN infected roots (A) and microscopic (10x) view of cyst in the root (B). Source: Owen Wally, Harrow Research and Developmental Centre-AAFC.

2.2 Nematode and plant CK profiling by mass spectrometry

2.2.1 CK extraction

Extraction and purification of CK were carried out using modified protocols described by Quesnelle and Emery (2007) and Farrow and Emery (2012). All the samples including nematodes and roots were lyophilized. The freeze-dried samples were re-suspended in 1mL of the extraction buffer Bieleski#2 (Methanol: Water: Formic Acid; CH_3OH: H_2O: HCOOH [15:4:1, v/v/v]) , spiked with 10 ng of each of the deuterated internal standard CKs (OlchemIm Ltd., Olomouc, Czech Republic; Table 5) and homogenized at 4^0C by a RetschMM300 ball mill grinder (Haan, Germany) for 5 min at 25 Hz with sterile zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada). Since labeled cZ CKs are not commercially available, quantification and identification of these compounds was based off the recovery of labeled *trans*-isomers and relative retention times of unlabeled cZ CKs in standard runs. Samples were allowed to extract passively overnight at –20^{0}C. Following overnight extraction, all the samples were centrifuged (Thermo Scientific, Massachusetts, United States) at 10,000 RPM for 10 min and the supernatant collected in another 2 mL tube. Solids were re-extracted by adding 1 mL ice-cold Bieleski #2 buffer for 30 min at -20^{0}C. Solid waste was separated and discarded by centrifugation at 10,000 RPM for 10 min and accumulated supernatants were combined in the same 2 mL tube. The pooled supernatants were evaporated in a speed vacuum concentrator at 35^{0}C (Fisher Scientific Inc., New Hampshire, USA). Extraction residues were reconstituted in 1 mL 1M formic acid (HCOOH) (pH 1.4) to ensure complete protonation of all CKs. Each extract was purified on a mixed mode, reverse-phase, cation-exchange cartridge (MCX 6 cc; Canadian Life Sciences, Peterborough, Ontario, Canada). Cartridges were activated with 5 mL HPLC grade methanol (CH_3OH) and equilibrated with 5 mL 1M of HCOOH (pH 1.4). After equilibration, samples were loaded onto the cartridge and washed with 5 mL 1M HCOOH (pH 1.4). CKs were eluted based on their chemical properties. NTCKs were eluted with 5 mL of 0.35 M ammonium hydroxide (NH_4OH) followed by FBCKs and RBCKs were eluted with 5 mL 0.35 M NH_4OH in 60% CH_3OH. Samples were evaporated to dryness in a speed vacuum concentrator at 35^{0}C and stored at -20^{0}C.

NTCKs were reconstituted in 1 mL 0.1 M ethanolamine-HCl (pH 10.4) and dephosphorylated to form RBCKs using 3 units of bacterial alkaline phosphatase (12 µL) (New England Biolabs, alkaline phosphatase calf intestine, Whitby, Ontario, Canada) for 12 hours at 37^{0}C (Emery et al., 2000). Resulting RBCKs were brought to dryness in a speed vacuum concentrator at 35^{0}C. The dephosphorylated nucleotides were reconstituted in 1.5 mL double distilled water for further purification on a reverse-phase C18 column (Canadian Life Sciences; C18/14, 3cc, 500mg; Peterborough, Ontario, Canada). Columns were activated using 3 mL of HPLC grade CH_3OH and equilibrated with 6 mL double distilled water. Samples were loaded onto the C18 column and allowed to pass through the column by gravity. The sorbent was washed with 3 mL double distilled water and analytes were eluted using 1.25 mL of 80% CH_3OH: H2O (80:20 v/v). All sample eluants were evaporated in a speed vacuum concentrator at 35^0C and stored at -20^0C until further processing.

FBCKs were resuspended and dissolved in 0.5 mL HPLC grade CH_3OH and transferred to 2 mL tubes; this was repeated two more times and the collected MeOH solution was evaporated at 35^0C in a speed vacuum concentrator. Prior to LC-MS/MS analysis, all purified CK fractions were reconstituted in 1.5 mL starting conditions buffer and later concentrated to 100 µL. 0.08% acetic acid (CH_3COOH) in 5% acetonitrile (ACN) were used as starting condition buffer. Samples were transferred to vial inserts and placed into glass HPLC vials and stored at -20^0C until analysis.

2.2.2 CK quantification by HPLC-MS/MS

Hormones were identified and quantified by HPLC-MS/MS analysis. A 25 µL aliquot of the sample volume was injected into a Dionex Ultimate 3000 HPLC coupled to a Q-Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Scientific, Massachusetts, United States). Compounds were resolved using a reversed-phase C18 column (Kinetex

2.6u C18 100 A, 2.1 × 50 mm; Phenomenex, Torrance, California, USA). All CK fractions were eluted with multistep gradient of component A: H_2O with 0.08% CH_3COOH mixed with component B: CH_3CN with 0.08% CH_3COOH at a flow rate of 0.4 mL min^{-1}. The initial conditions were 5% B increasing linearly to 10% B over 2 minutes followed by an increase to 95% B over 6.5 min; 95% B was held constant for 1.5 minutes before returning to starting conditions for 5 minutes. The effluent was introduced into the Orbitrap electrospray source (capillary temperature of 250°C) and the hormone samples were identified by parallel reaction monitoring (PRM) of the ionized intact molecules at 35,000 resolution. CKs were analyzed in positive $[M+H]^+$ ionization modes. The HESI source was operated with sheath gas, 30 arbitrary units; auxiliary gas, 8 arbitrary units; max spray current, 100A; probe temperature, 450°C; and S-lens RF level, 60. Spray voltage was 3.9 kV and 3.5kV in the positive mode. The PRM parameters included: automatic gain control (AGC), $3×10^6$; maximum injection time (IT), 128 ms; 1.2 m/z isolation window and 35 normalized collision energy (NCE).

Table 5: Cytokinins (CKs), scanned for by liquid chromatography-positive electrospray ionization tandem mass spectrometry (HPLC- (ESI+)-MS/MS).

	Isoprenoid cytokinins	Labeled CK standard
	Nucleotides (NT)	
1.	*trans*-zeatin riboside-5'-monophosphate (*t*ZNT)	$^{2}H_{5}$[9RMP]Z
2.	*cis*-zeatin riboside-5'-monophosphate (*c*ZNT)	
3.	Dihydrozeatin riboside -5'-monophosphate (DHZNT)	$^{2}H_{3}$[9RMP]DZ
4.	*N*^{6}-isopentyladenosine-5'monophosphate (iPNT)	$^{2}H_{6}$[9RMP]iP
	Ribosides (RB)	
5.	*trans*-zeatin riboside (*t*ZR)	$^{2}H_{5}$[9R]Z
6.	*cis*-zeatin riboside (*c*ZR)	
7.	Dihydrozeatin riboside (DHZR)	$^{2}H_{3}$[9R]DHZ
8.	*N*^{6}-isopentyladenosine (iPR)	$^{2}H_{6}$[9R]iP
	Free bases (FB)	
9.	*trans*-zeatin (*t*Z)	
10.	*cis*-zeatin (*c*Z)	$^{2}H_{3}$DHZ
11.	Dihydrozeatin (DHZ)	
12.	*N*^{6}-isopentyladenine (iP)	$^{2}H_{6}$iP
	Glucosides (Gluc)	
13.	*trans*-zeatin-O-glucoside (*t*ZOG)	$^{2}H_{5}$ZOG

14.	*cis*-zeatin-O-glucoside (*c*ZOG)	
15.	Dihydrozeatin-O-glucoside (DHZOG)	$^{2}H_{7}$DHZOG
16.	*trans*-zeatin-O-glucoside riboside (*t*ZROG)	$^{2}H_{5}$ZROG
17.	*cis*-zeatin-O-glucoside riboside *c*ZROG	
18.	Dihydrozeatin-O-glucoside riboside (DHZROG)	$^{2}H_{7}$DHZROG
19.	*trans*-zeatin-9-glucoside (*t*Z9G)	$^{2}H_{5}$Z9G
20.	*cis*-zeatin-9-glucoside (*c*Z9G)	
21.	Dihydrozeatin-9-glucoside (DHZ9G)	$^{2}H_{3}$DHZ9G
22.	*trans*-zeatin-9-glucoside (*t*Z7G)	$^{2}H_{5}$Z7G
	Methylthiols (2MeS-CKs)	
23.	2-Methylthio-*trans*-zeatin (2MeSZ)	$^{2}H_{5}$MeSZ
24.	2- Methylthio-*trans*-zeatin riboside (2MeSZR)	$^{2}H_{5}$MeSZR
25.	2-Methylthio-N^{6}-isopentyladenine (2MeSiP)	$^{2}H_{6}$MeSiP
26.	2-Methylthio-N^{6}-isopentyladenosine (2MeSiPA)	$^{2}H_{6}$MeSiPR
	Aromatic cytokinins	Labeled CK standard
27.	Benzyloaminopurine (BA)	$^{2}H_{7}$BA
28.	Benzyloaminopurine riboside (BAR)	$^{2}H_{7}$BAR

2.2.3 Data analysis

All hormone data acquisition, processing and quantification were completed with Thermo Xcalibur 4 with foundation 3.0 SP2 software (Thermo Scientific, Massachusetts, USA). Quantification was achieved through the isotope dilution analysis based on recovery of ^{2}H-labelled internal standards.

Soybean root hormone profiles were grouped based on the SCN treatment to the cultivars and SCN susceptibility at each developmental stage. The two species of nematodes hormone profile were grouped on their developmental stage. Four independent biological replicates were used for soybean roots (n=4) and three independent biological replicates (n=3) were used for each species/stage of nematodes. Endogenous hormone profiles were analyzed using Prism (v 6.0, GraphPad Software, Inc., San Diego, California, USA). Statistical analysis was carried out using a two-way ANOVA with the Duncan's multiple range test (MRT) in Statistica (v 8.0, TIBCO Software Inc. Palo Alto, California, USA). Significant differences between the treatments refer to a p-value of <0.05. The results of significance tests were added to the tables of detailed CK levels in the appendix.

3 Results

3.1 Comparative analysis of endogenous CK profiles of *C. elegans* and *H. glycines*

Total CKs of *C. elegans* and *H. glycines* (SCN) were extracted and analyzed at two developmental stages: egg and larva. All data were normalized and presented as picomole per nematode (pmol nematode^{-1}).

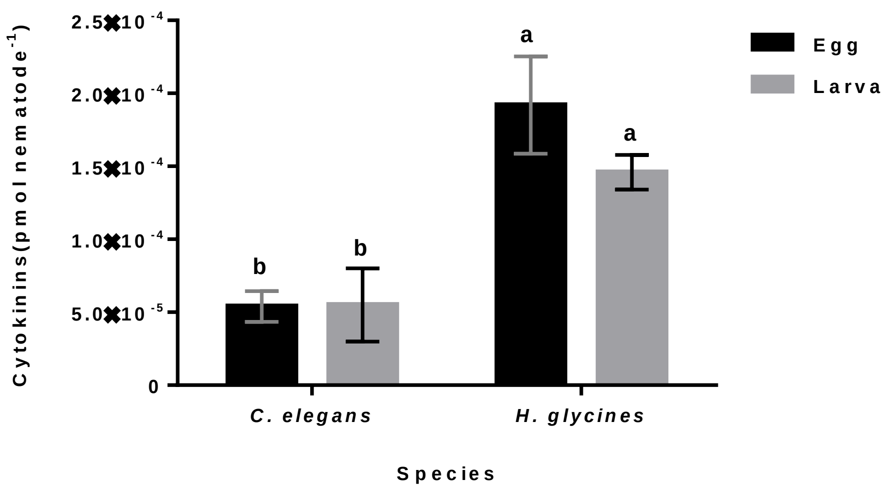


Figure 11- Total CK level (pmol nematode^{-1}) in *C. elegans* and *H. glycines* at two developmental stages; egg and larva. Bars represent mean ± SE for 3 independent biological replicates. Data were analyzed using two-way ANOVA ($P < 0.05$) and Duncan post hoc test. Values that do not share the same letter are significantly different from each other.

As depicted in figure 11, the overall CK concentration in SCN was significantly higher than that of *C. elegans* at both developmental stages. FBCKs and NTCKs comprised the major CK pool in *C. elegans* and SCN, respectively (figure 12 A-D). Levels of each CK group in *C. elegans* decreased in larva stage except for NTCKs which increased approximately 3-fold at larval stage (10% to 33% of total CKs) (figure 12 A-B). In contrast, both SCN egg and larva were mainly composed of NTCKs, levels of which decreased at larva stage (88% of total CKs in egg and 75% in larva) (figure 12 C, D). Similarly, the RBCK level was lower in the SCN larval stage compared to the eggs (4% and 0.5% of total CKs, respectively). The levels of FBCKs did not change in SCN (2% of total CKs in both stages). The levels of 2MeSCKs increased from 5% to 23% of total CKs from SCN egg to larval stage (figure 12 C, D). Glucosides were detected only at trace levels at both developmental stages in the two tested species of nematodes. All the data are obtained from table 8.6 in the appendix.

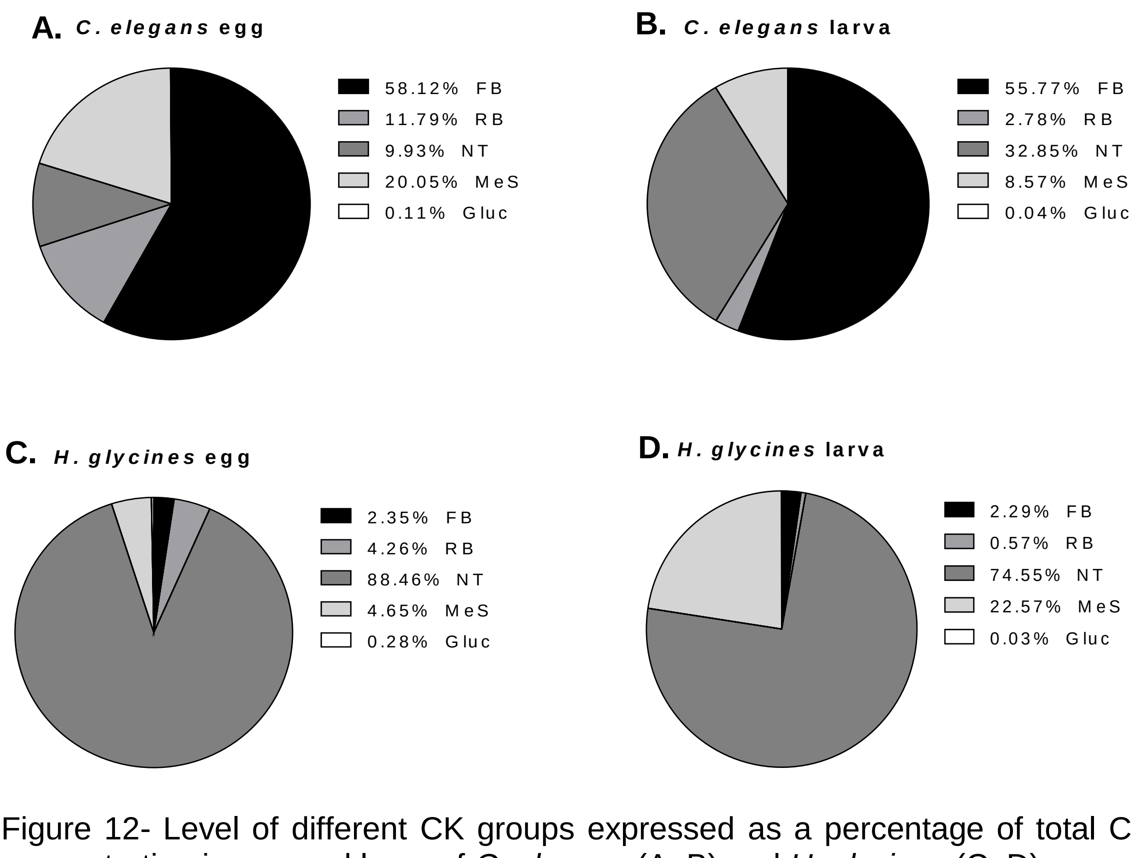


Figure 12- Level of different CK groups expressed as a percentage of total CK concentration in egg and larva of *C. elegans* (A, B) and *H. glycines* (C, D).

Of the 28 endogenous CKs monitored, 22 were detected in *C. elegans* and SCN eggs and larvae (Appendix, table 8.1). CK hormones in *C. elegans* and SCN are further discussed below, separated into different groups (free bases, ribosides, nucleotides and methylthiols).

Free bases

FBCK profiles were different between *C. elegans* and SCN. They were the most abundant group detected in *C. elegans* egg and larva (figure 12 A, B) and their levels were significantly higher than in SCN. In both stages, *C. elegans* contained the highest concentration of iP freebase (iP), while this was the second most abundant type of CK in SCN (figure 13 A). In SCN, cZ freebase (cZ) was the dominant CK type at both developmental stages and the second most abundant CK in *C. elegans*. Both species showed a decrease in cZ over their developmental stages, whereas no significant change in iP was found. Furthermore, *C. elegans* contained dihydrozeatin freebase (DHZ) and SCN contained tZ freebase (tZ) in trace concentrations.

Ribosides

RBCKs were detected in *C. elegans* and SCN eggs and larvae in the concentrations not exceeding 12% of total CKs (figure 12 A-D). The RBCK profiles were different between *C. elegans* and SCN. All types of RBCKs were detected in *C. elegans* at various levels, while in SCN DHZ riboside (DHZR) and iP riboside (iPR) were detected the highest concentration with a trace amount of cZ riboside (cZR) (figure 13 B). tZ riboside (tZR) was not detected in any developmental stages of SCN.

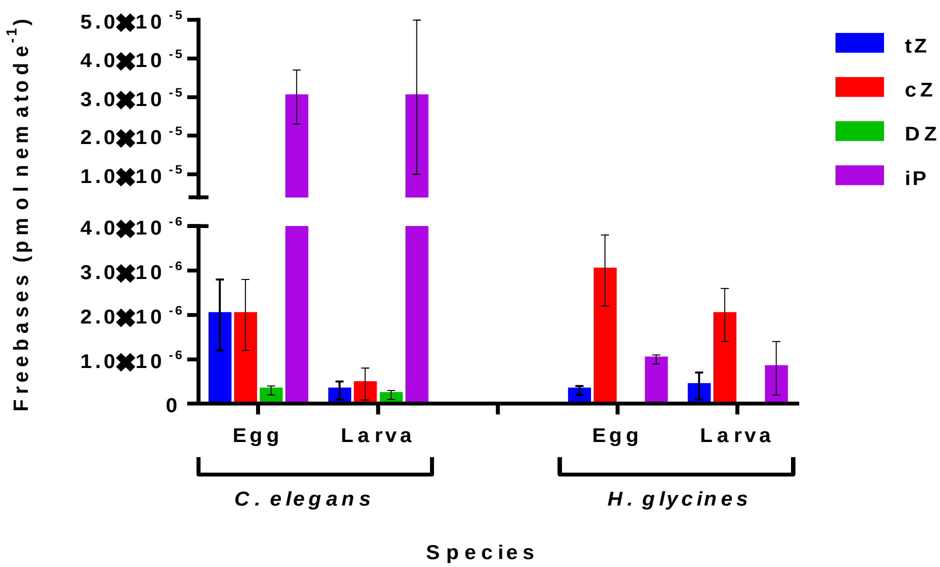
Nucleotides

Overall, *C. elegans* and SCN showed similar patterns regarding their nucleotide profiles. The majority of CK pool in SCN was comprised of NTCKs which was the second most abundant group in *C. elegans* larva (figure 12 B-D). Eggs and larvae of *C. elegans* and SCN contained iP nucleotide (iPNT) at the highest concentration, representing almost 100% of CKs found in SCN (figure 13 C). The level of iPNT was relatively stable from egg to larva of both nematodes. Low levels of tZ nucleotide (tZNT) and cZ nucleotide (cZNT) were also detected in *C. elegans* egg and larva while these CKs were found in SCN egg in trace concentrations with none being detected in the larva.

Methylthiols

Profiles of 2MeSCKs were relatively similar between nematode species and between egg and larva within species. They were the second most abundant CK group in *C. elegans* eggs, whereas third in larva stage, behind FBCKs and NTCKs (figure 12 A-B). Similarly, in SCN egg and larva, 2MeSCKs were the second most abundant CK group identified (figure 12 C-D). The levels of 2MeSCKs detected in *C. elegans* and SCN were mainly composed of 2-methylthio-trans-zeatin (2MeSZ) and 2-methylthiol-N6-isopentenyladenine (2MeSiP), where both hormone levels decreased in *C. elegans* larva but increased in SCN (figure 13 D) compared to the corresponding egg levels. Relatively lower levels of 2-methylthio-trans-zeatin riboside (2MeSZR) and 2-methylthio-N^6-isopentenyladenosine (2MeSiPR) were found in eggs and larvae of both nematodes, where at larva stage both hormone levels decreased.

A.



B.

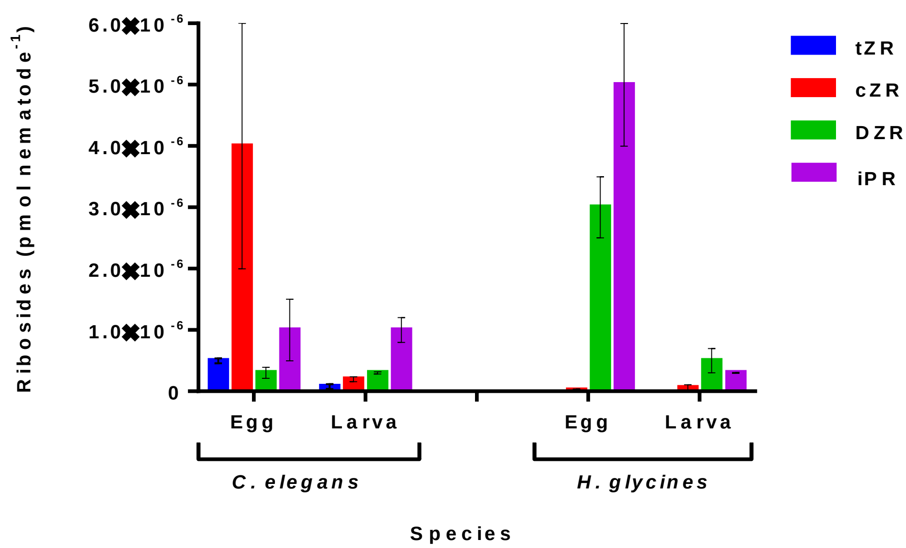
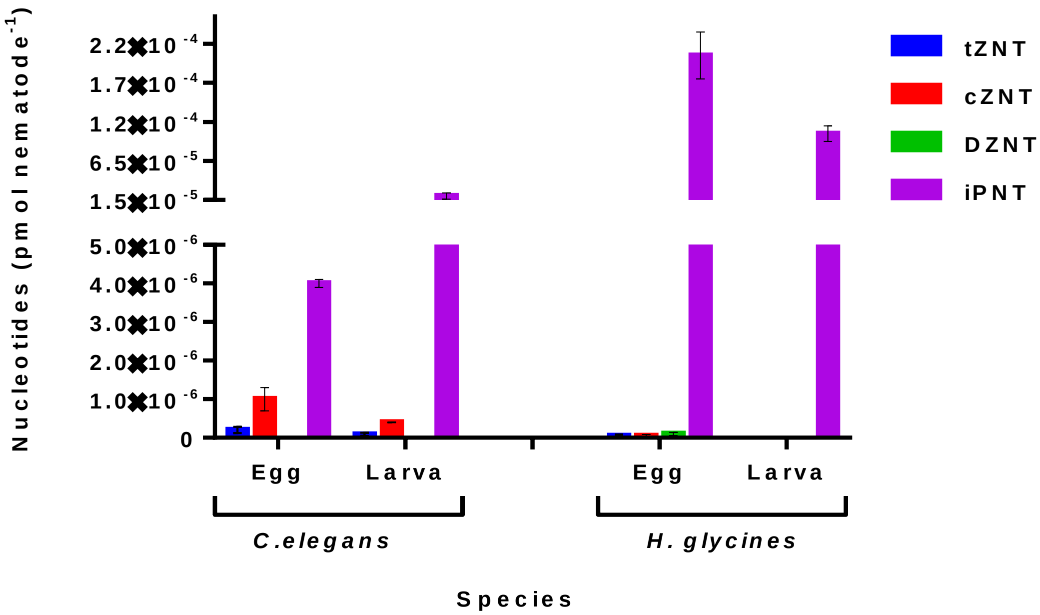
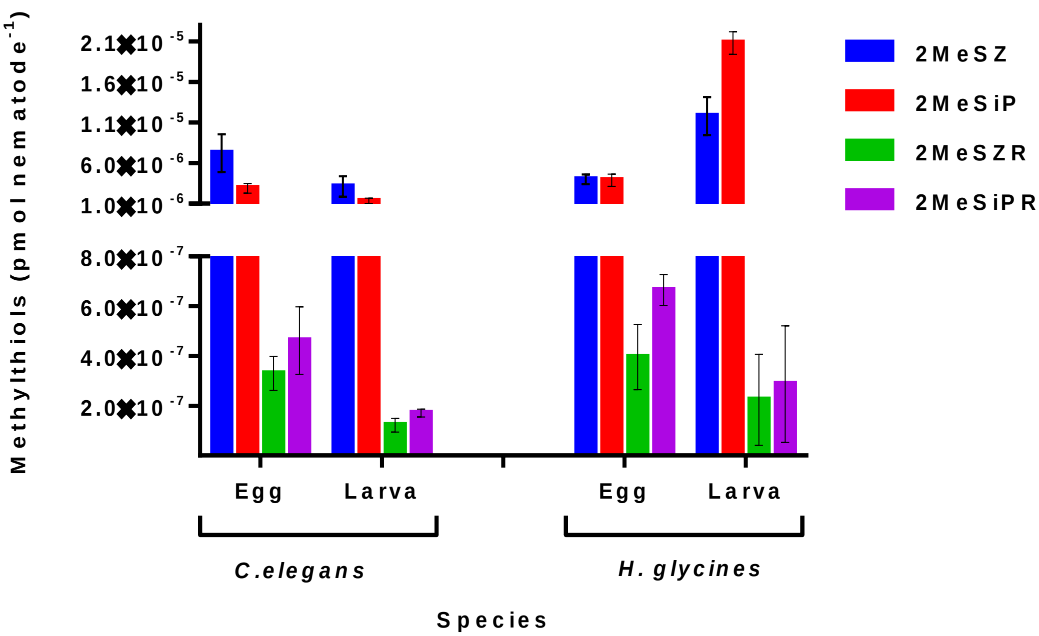


Figure 13- The endogenous cytokinin content separated into freebases (A), ribosides (B), nucleotides (C) and methylthiols (D) in eggs and larvae of *C. elegans* and *H. glycines*. Bars represent mean ± SE for 3 independent biological replicates.

C.


Nucleotides (pmol nematode^{-1})
2.2×10^{-4}
1.7×10^{-4}
1.2×10^{-4}
6.5×10^{-5}
1.5×10^{-5}
5.0×10^{-6}
4.0×10^{-6}
3.0×10^{-6}
2.0×10^{-6}
1.0×10^{-6}
0
tZNT
cZNT
DZNT
iPNT
Egg
Larva
Egg
Larva
C.elegans
H. glycines
Species

D.


Methylthiols (pmol nematode^{-1})
2.1×10^{-5}
1.6×10^{-5}
1.1×10^{-5}
6.0×10^{-6}
1.0×10^{-6}
8.0×10^{-7}
6.0×10^{-7}
4.0×10^{-7}
2.0×10^{-7}
2MeSZ
2MeSiP
2MeSZR
2MeSiPR
Egg
Larva
Egg
Larva
C.elegans
H. glycines
Species

3.2 Validation of SCN resistance in soybean cultivars

The two SCN susceptible and resistant soybean cultivars (samples 1,2 & 5,6 respectively) were selected for analysis. The SCN genotyping confirmed the SCN resistance in the two selected resistant cultivars. The PCR products at 200 bp were the lectin gene which was used as internal control in this experiment (figure 14). The gene responsible for SCN resistance (*rhg 1*) in the two resistant cultivars was amplified as a 400 bp fragment and its presence confirmed their SCN resistance.

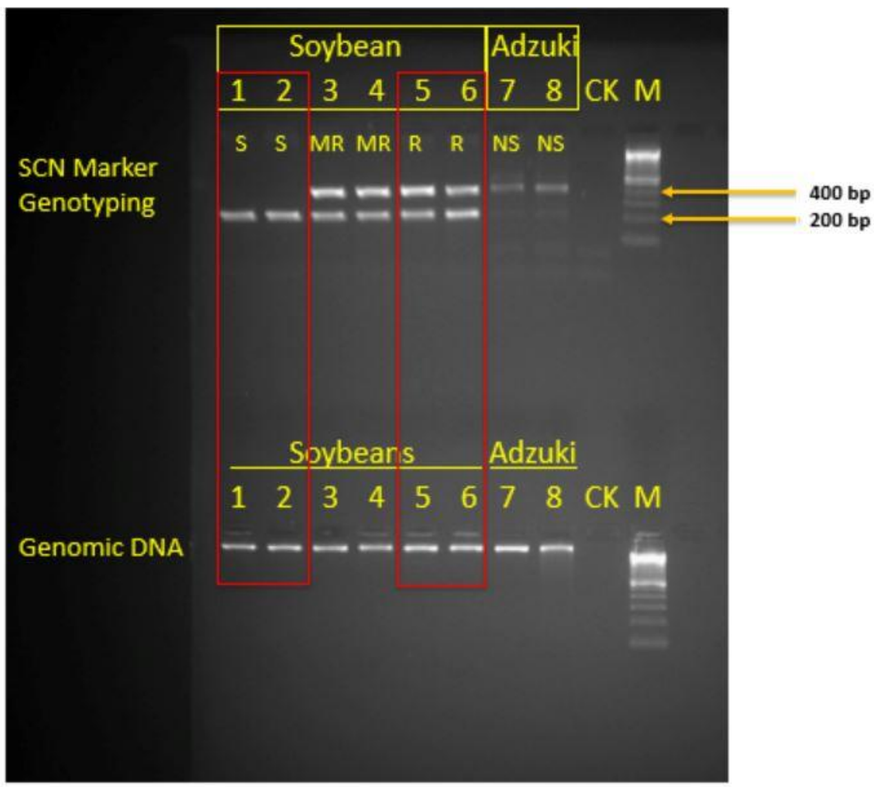


Figure 14- SCN genotyping results. Legend: S - susceptible cultivars (1; Lee74, 2; Essex), R - resistant cultivars (4; Pion 22T69R, 5; Pion 93Y05), CK – blank control (check), M – DNA size mass ladder (1Kb DNA Ladder, Invitrogen). The remaining lanes contain samples that were not analyzed in this thesis. There were 3 biological replicates for each cultivar and 2 technical replicates for each sample.

3.3 Endogenous CK profiles in soybean roots at different developmental stages

Four soybean cultivars analyzed for their endogenous CK profiles, including SCN susceptible (Lee74, Essex) and resistant (Pion 22T69R and Pion 93Y05) cultivars, were grown in the greenhouse and roots were collected at three plant developmental stages: vegetative emergence stage (VE) (5DAS), first trifoliate stage or vegetative stage 1 (V1) (14DAS) and beginning bloom or reproductive stage 1 (R1) (40DAS) to extract and profile endogenous CKs by HPLC-MS/MS. All data were normalized and presented as picomole per gram dry weight of the root tissue (pmol g^{-1}DWt). The roots at VE stage (5DAS) were mainly analyzed to determine the correlation between SCN resistance and CK production in non-infected soybean plants. So, a comparative analysis of endogenous CK profiles of SCN susceptible and SCN resistant cultivars was conducted with no SCN inoculation. Soybean plants that were grown for V1 (14 DAS) and R1 (40DAS) stages, were inoculated with SCN eggs (4000 eggs/plant) during seed sowing and the roots were collected and analyzed to determine altered CK dynamics in soybean cultivars both before, and in response to, SCN infection.

CK concentrations varied throughout the three soybean developmental stages. As shown in figure 15 A, the differences in total CK levels between the two groups of soybean cultivars (SCN susceptible vs. resistant) were not significant at VE stage. Total CK levels of all four soybean cultivars increased (significant for Essex and Pion 22T69R) in response to SCN infection except for Pion 93Y05 at V1 stage (figure 15 B). Noticeably, the levels of total CK in SCN resistant cultivar; Pion

93Y05, were the highest of all other non-infected roots (figure 15 B, $P<0.05$) which was comparable to the infected root CK levels seen in the other three cultivars at V1 stage. Interestingly, the total CKs in Pion 93Y05 did not increase in response to SCN infection (figure 15 B). However, at R1 stage the same cultivar showed a significant increase in total CKs in response to SCN infection at a reduced concentration level of CKs (figure 15 C). Finally, the two SCN susceptible cultivars; Lee74 and Essex behaved similarly in response to SCN infection at V1 and R1 stage, showing a significant increase of total CKs in Essex only (figure 15 B-C). Changes in soybean CK hormone levels caused by root infection with SCN are further discussed below, separated into different groups (free bases, ribosides, nucleotides, methylthiols and glucosides).

Free bases

FBCKs in the soybean cultivars at VE stage showed no significant difference between SCN susceptible and resistant cultivars (figure 16 B). At V1 stage, FBCKs comprised the major CK pool in the soybean cultivars (figure 17 A) with iP being the dominant type (80-100%) (figure 17 C). Interestingly, SCN infection caused a significant increase of FBCK production in all the analyzed cultivars except for Pion 93Y05. This particular cultivar did not respond to the infection (figure 17 C, $P<0.05$). A closer look at the individual CK types reveals what was behind the plant response to SCN and the differences observed for Pion 93Y05. Unlike V1 stage, increases of FBCKs level at R1 stage were mostly caused by large increases in cZ (figure 17 D). Interestingly, the susceptible cultivars (Lee74 and Essex) showed much greater cZ increase due to infection, compared to Pion 22T69R or Pion 93Y05.

Ribosides

Similar to FBCKs, RBCKs in the soybean cultivars at VE stage showed no significant difference between SCN susceptible and resistant cultivars (figure 16 C). SCN infection caused a significant increase of RBCKs in soybean cultivars at V1 stage (figure 17 E) where all the RBCK forms were increased except DHZR. Like in the V1 stage, the levels of RBCKs were also significantly increased at R1 stage where tZR was the most abundant CK type detected in SCN infected roots (figure 17 F).

Nucleotides

Similar to RBCKs, no significant difference of NTCKs was observed between SCN susceptible and resistant cultivars at VE stage (figure 16 D). The levels of NTCKs in soybeans at V1 stage were increased in the SCN infected roots (significant difference in Lee74 and Pion 93Y05)and iPNT was the most abundant CK type detected for both SCN non-infected and infected roots (figure 17 G). At R1 stage, NTCKs were highly detected in the SCN infected roots and tZNT was found the most abundant NTCK in the infected roots of Lee74 and Essex (SCN susceptible cultivars), whereas iPNT was highly detected in Pion 22T69R and Pion 93Y05 (SCN resistant cultivars) (figure 17 H).

Methylthiols and glucosides

There was no significant change of total level of 2MeSCKs and glucosides observed between SCN susceptible and resistant cultivars at VE stage (figure 16 E, F). Interestingly, 2MeSZR was detected significantly higher in the two SCN resistant cultivars than the SCN susceptible cultivars (figure 16 E). In response to SCN infection, the soybean cultivars at V1 and R1 stages showed no significant change in the level of 2MeSCKs, although 2MeSZ and 2MeSiP were highly detected at both stages (figure 17 I, J). The level of glucosides was the lowest compared to all other CK groups regardless of the developmental stages. The only significant changes in glucoside levels were observed in Essex at V1 and Lee74 at R1 stage (figure 17 K, L).

A. Vegetative emergence stage (VE)

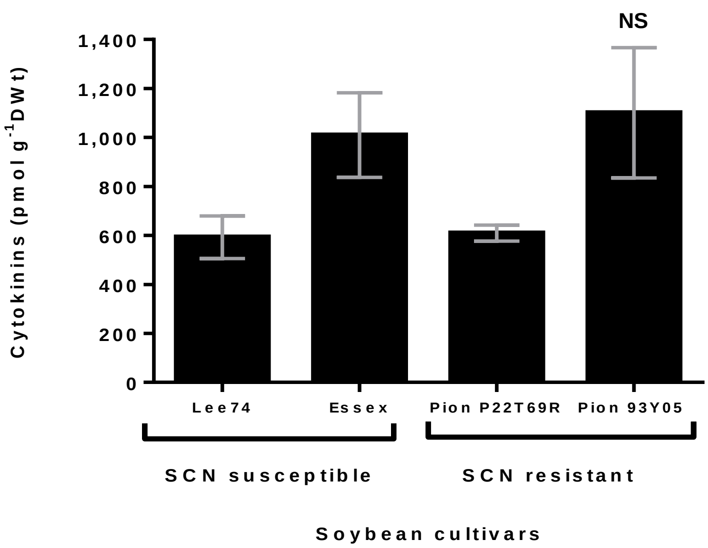
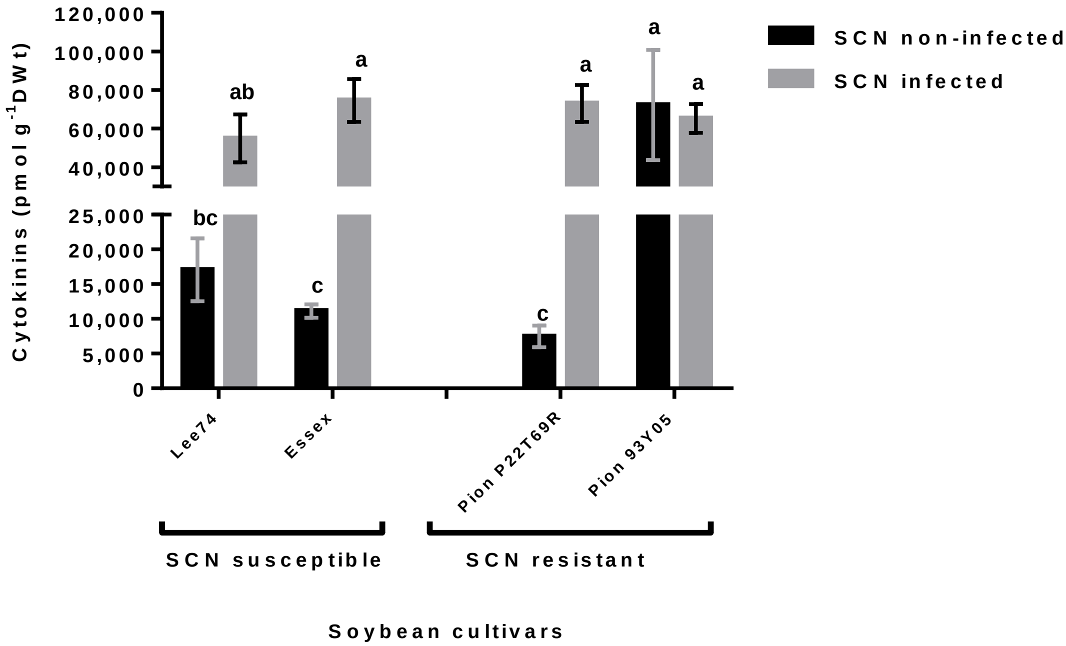
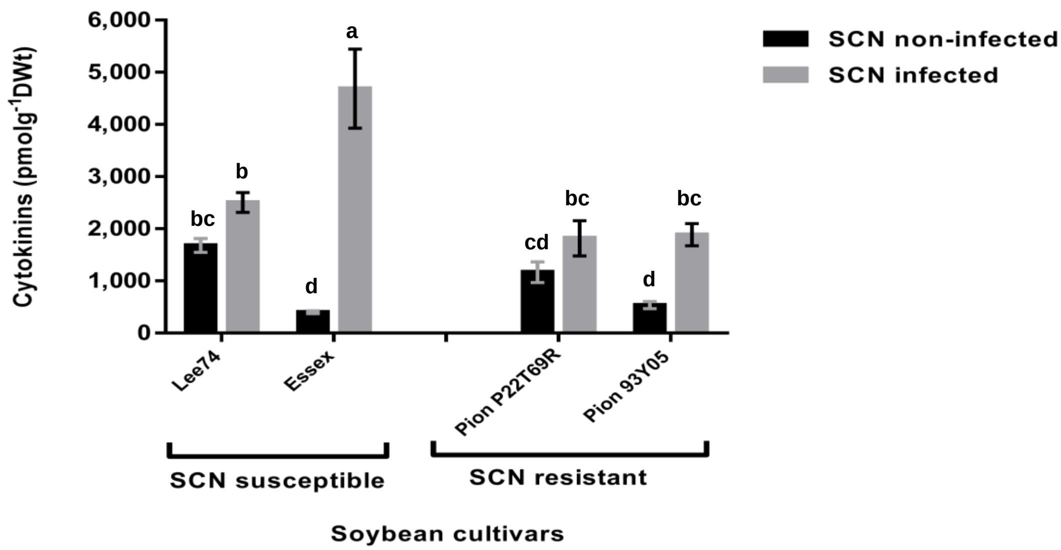


Figure 15- Total cytokinins in soybean roots at three developmental stages (A, VE, B, V1 and C, R1). Bars represent mean ± SE for 4 independent biological replicates. Data were analyzed using two-way ANOVA ($P < 0.05$) and Duncan post hoc test. Values that do not share the same letter are significantly different from each other.

B. Vegetative stage 1 (V1)



C. Reproductive stage 1 (R1)



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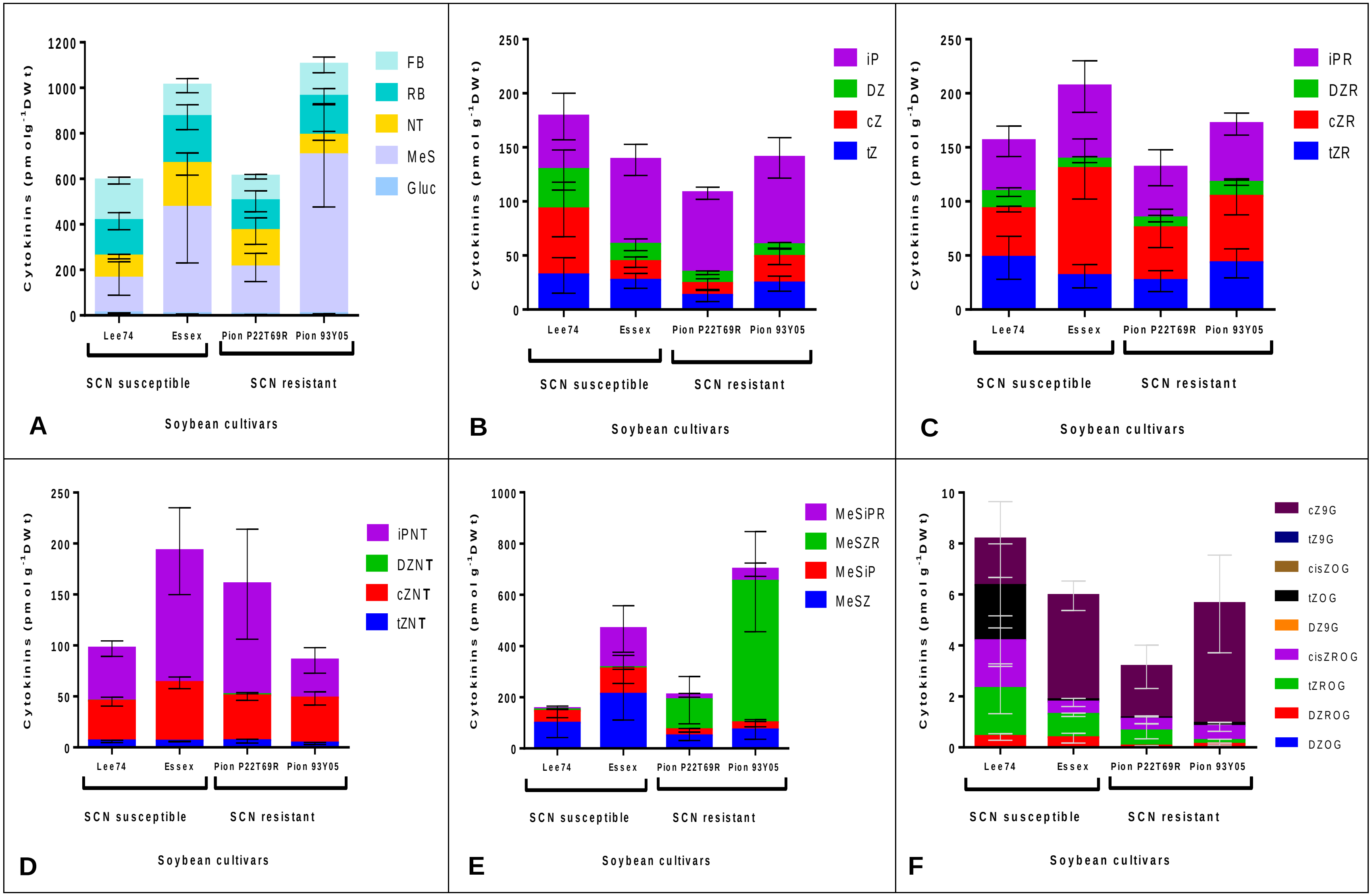
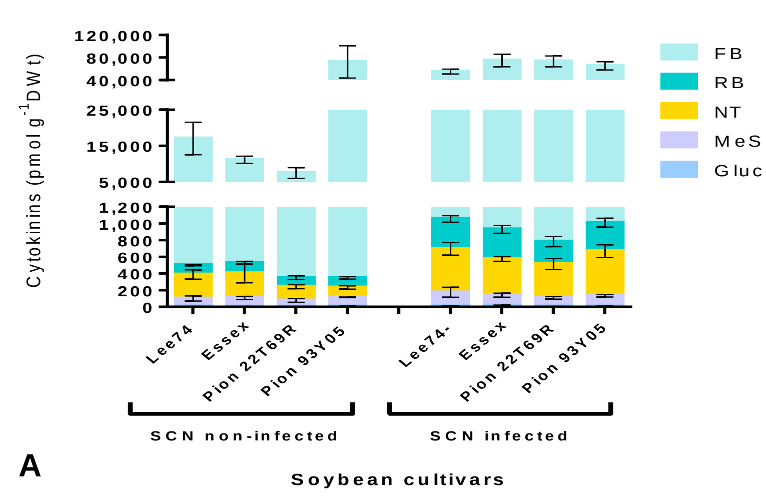
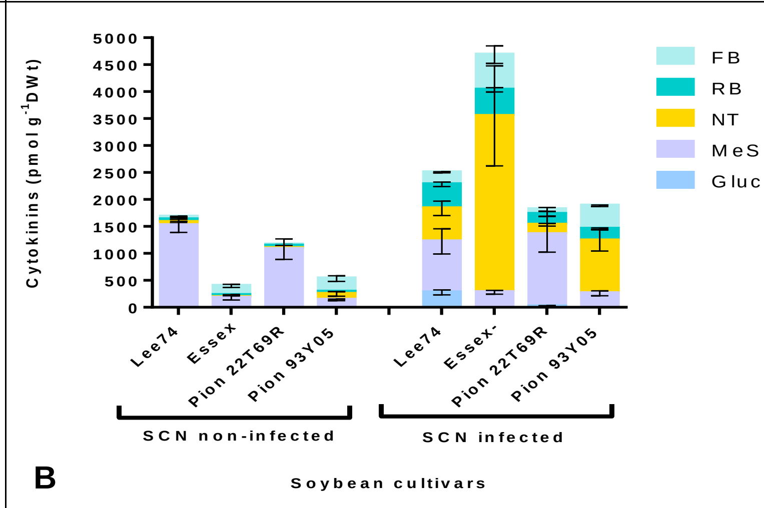
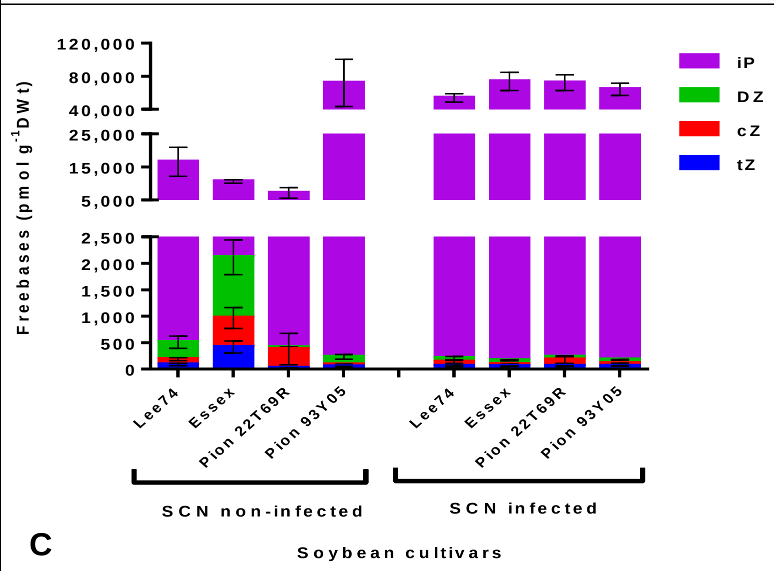
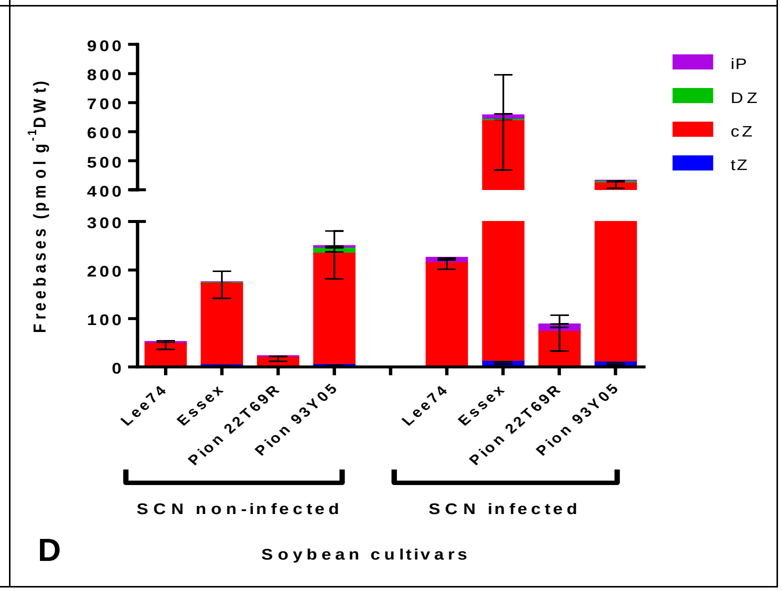
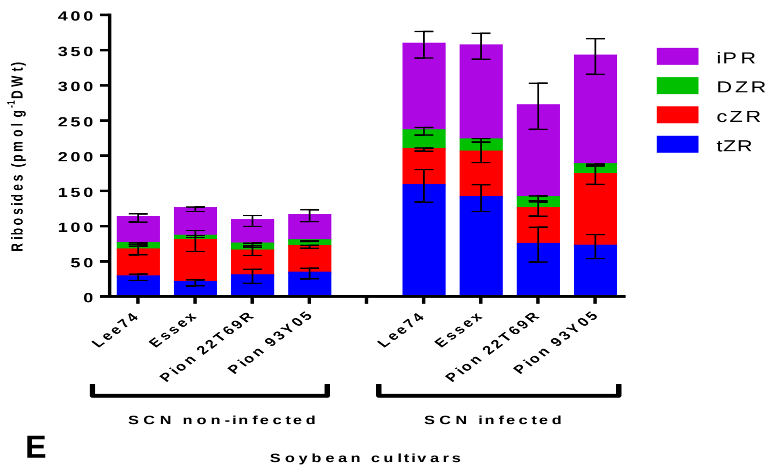
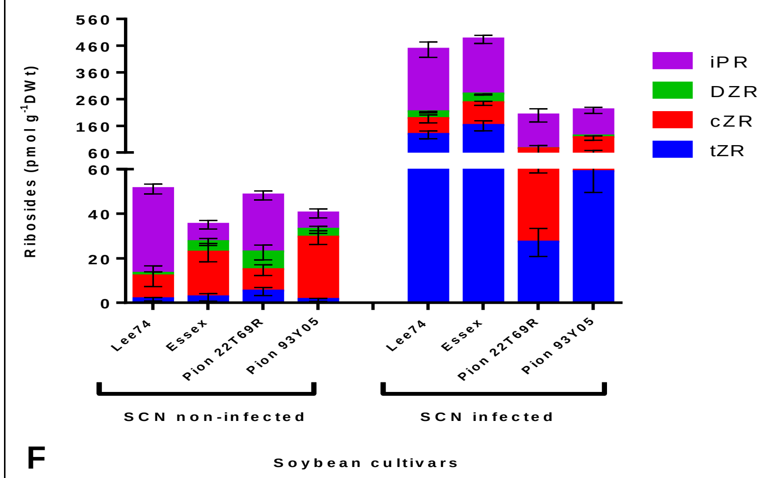
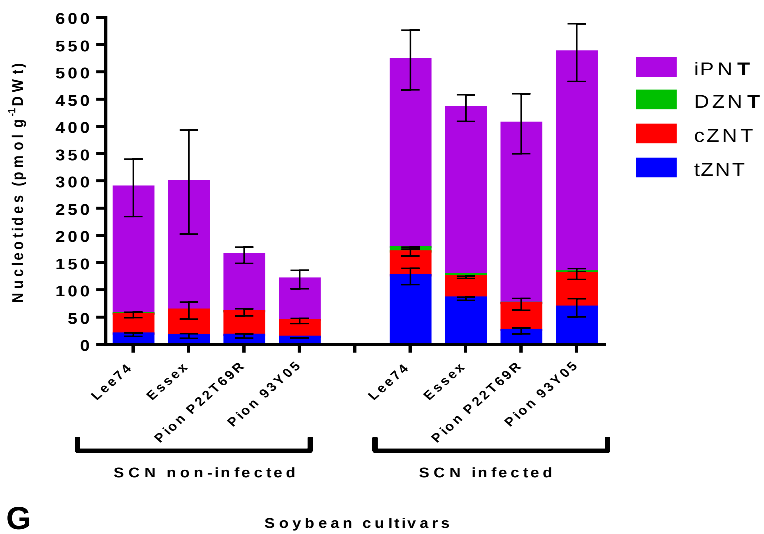
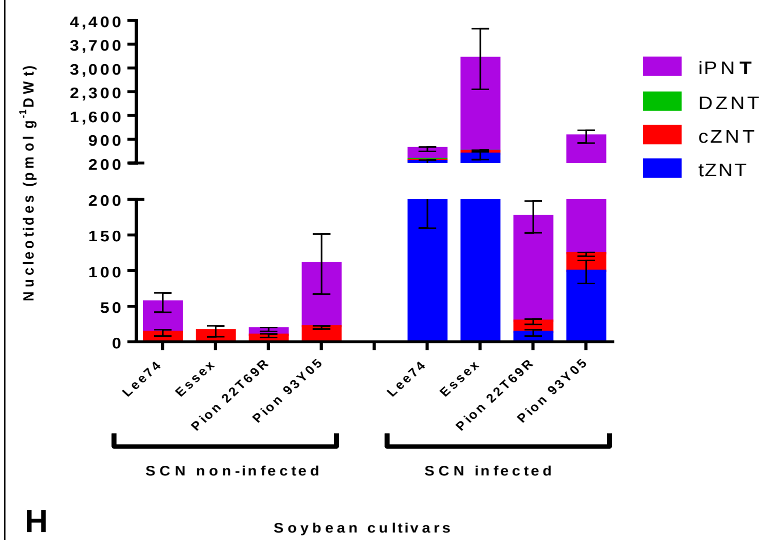


Figure 16- Average endogenous cytokinin content (pmol g^{-1}DWt) in soybean at vegetative emergence (VE) stages. CK groups (A), free bases (B), ribosides (C), nucleotides (D), methylthiols (E), glucosides (F). Bars represent mean ± SE for 3 independent biological replicates.

	Vegetative stage 1 (V1)	Reproductive stage 1 (R1)
Total-CK groups		
FBCKs		

	Vegetative stage 1 (V1)	Reproductive stage 1 (R1)
RBCKs		
NTCKs		

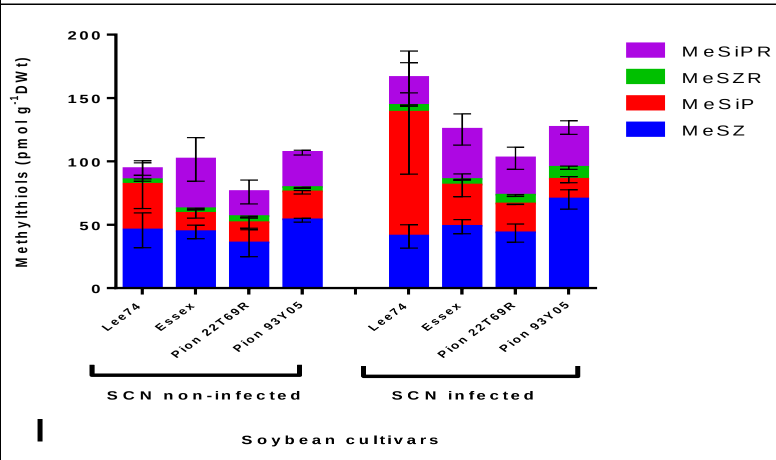
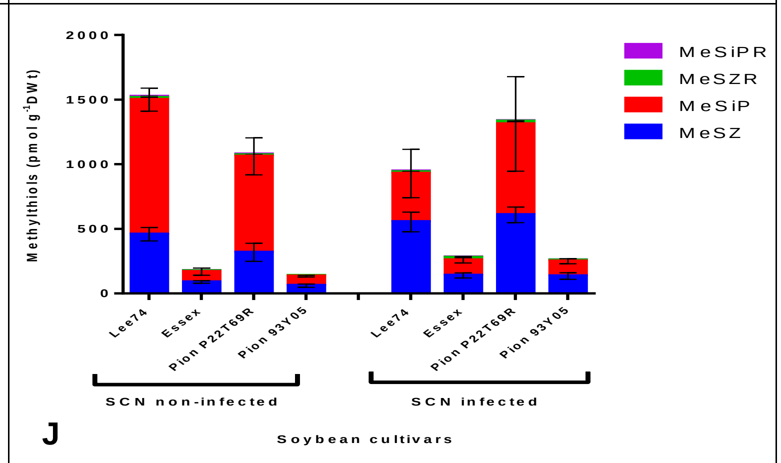
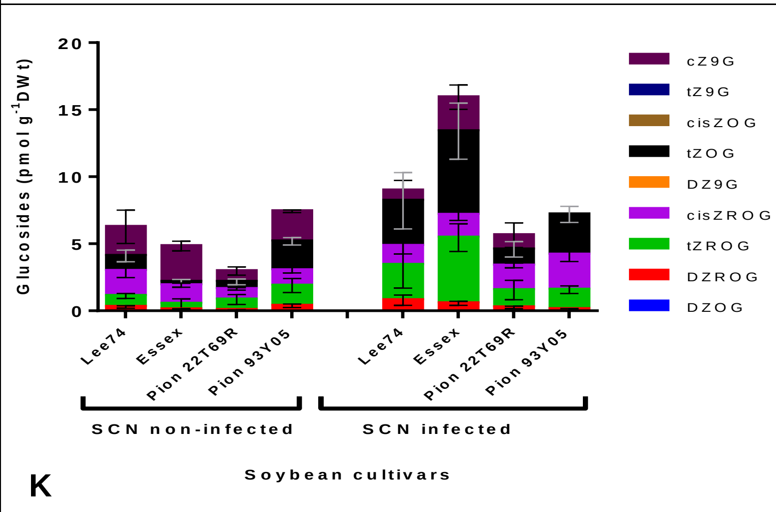
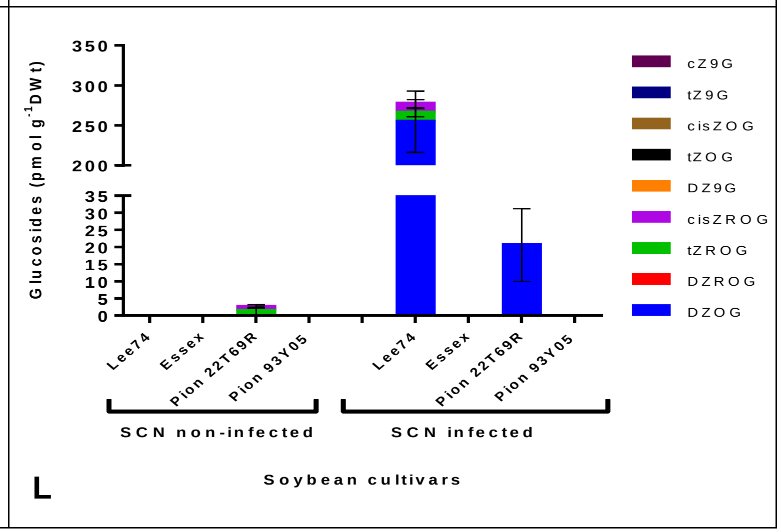
	Vegetative stage 1 (V1)	Reproductive stage 1 (R1)
2MeS-CKs		
Gluc		

Figure 17- Average endogenous cytokinin content (pmol g^{-1}DWt) in SCN non-infected (control) and infected plants of four soybean cultivars at two developmental stages, vegetative stage 1 (V1) or first trifoliate stage and reproductive stage 1 (R1) or beginning bloom. Bars represent mean ± SE for 4 independent biological replicates.

4 Discussion

4.1 General overview

Nematodes are a major component of soil and sediment ecosystems with both beneficial and harmful effects. Numerous studies have been conducted on nematodes and their interactions with other organism but not much is known about the roles of CKs in nematodes. This thesis research conducted the first extensive examination of CK activity profiles in nematodes and tested nematode originated CK influence on host plants pathogenesis. To do this, I analyzed eggs and larvae of soil free living *C. elegans* and a plant parasitic *H. glycines* (soybean cyst nematode; SCN) along with two SCN susceptible (Lee74 and Essex) and two resistant cultivars (Pion 22T69R and Pion 93Y05) of soybean at different developmental stages. The efficient techniques of hormone extraction and considerably greater sensitivity in mass spectrometry (Farrow and Emery, 2012) allowed me to determine the profiles of 28 different CK compounds, out of which 22 were detected in nematode samples at different concentrations. The current study reported that *C. elegans* and *H. glycines* are able to synthesize a broad range of CKs including iP, cZ, tZ and DZ, as well as their corresponding riboside and nucleotide forms. Notably, this is the first study to report the synthesis of methylthiolated CK conjugates in nematodes. It was hypothesized in the first part of the thesis that CK profiles of *C. elegans* and *H. glycines* would be different. More specifically, I predicted that CK profiles of *C. elegans* and *H. glycines* eggs would be similar as no parasitism occurs at this stage, but profiles would be different at the larval stages of these nematode species. Based on the earlier reports, it was further predicted that the larva of *H. glycines* would have a more active CK profile composed of higher level of CKs that are known for high biological activity in plants (i.e. free bases) than *C. elegans* larva, as it is the only infective stage of *H. glycines*. The results showed that level of total CKs of *C. elegans* and *H. glycines* were significantly different at both egg and larvae stages. However, CKs in *H. glycines* were 93% (egg) and 97% (larva) in precursor forms (biologically inactive in plant), whereas in *C. elegans*, 70% and 58% respectively, were in highly bioactive forms (free bases and ribosides). So, the hypothesis, that SCN would have a more active CK profile, was rejected by the obtained data and it was suggested that CKs are present in nematodes to regulate endogenous function in nematode physiology and *H. glycines* may convert inactive to active CKs during infection only. 2MeSCKs were found abundant in both egg and larvae of *C. elegans* and *H. glycines*. Interestingly, the level of 2MeSCKs was 5-fold higher in *H. glycines* larva (the only infectious stage of SCN) compare to their egg and 3-fold higher than *C. elegans* larva, indicating a potential role for 2MeSCKs in *H. glycines* infection of plants. CKs were identified as one of the key compounds in previous reports on Arabidopsis-*H. schachtii* and Arabidopsis-*M. incognita* interactions. In contrast to earlier findings, however, it has never been evaluated in soybean (*Glycine max*)-*H. glycines* system. So, in the second part of the thesis, I tested the never evaluated hypothesis that SCN resistance in soybean (*Glycine max* (L.) Merr.) is expressed through the altered plant CK dynamics both in control (SCN non-infected) and in response to SCN infection (SCN infected). The CK profiles of two SCN-susceptible (Lee74, Essex) and two resistant (Pion 22T69R and Pion 93Y05) cultivars at three developmental stages (VE, V1, and R1) revealed that regardless of the resistance level, SCN infection caused a significant increase in soybean root CKs. However, the increases were similar between the susceptible and resistant soybean cultivars except, one of the resistant cultivars, Pion 93Y05. Pion 93Y05 showed consistently high levels of a bioactive CK, iP in the non-infected roots, comparable with the increased upon SCN infection hormone levels in the roots of the remaining three cultivars. This indicates that the correlation between CK production and SCN resistance may exist in soybean.

4.2 Role of CKs in nematodes

The role of CKs in plants has been widely studied as CKs have been identified as the key phytohormones in many aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, apical dominance, sink/source relationships, nutrient uptake, embryonic development, as well as the response to biotic and abiotic factors (Mok & Mok, 2001; Kieber, 2002; Spíchal, 2012; Kieber & Schaller, 2014; Cortleven et al., 2018). Currently, the increasing evidence suggests a role for CKs in organisms beyond the plant kingdom such as bacteria and fungi and characterizes CKs as key regulatory molecules in plant biotic interactions (Kisiala et al., 2013; Giron et al., 2013; Morrison et al., 2017). The best-characterized microbial system for the production and role of CKs is the crown gall-forming gram-negative bacterium *Agrobacterium tumefaciens* which contains two CK biosynthesis IPT genes in its Ti-plasmid that integrates into plant genomes during infection and stimulates cell proliferation and gall formation (Sakakibara, 2006). In addition to bacteria, the plant pathogenic fungus *Ustilago maydis* has also been shown to produce and secrete CKs into the host plant for effective disease development (Morrison et al., 2017).

The ability of plant parasitic animals such as nematodes to produce CKs and use it to manipulate the plant metabolism was unknown for a long period of time. Recently, gene expression studies on Arabidopsis-*H. schachtii* and Arabidopsis-*M. incognita* reported that plant parasitic nematodes are able to synthesize plant hormones, CKs, to reprogram host root cells into unique metabolically active feeding sites (Siddique et al., 2015; Dowd et al., 2017; Siddique & Grundler, 2018). But the question raised was whether the amount of CKs secreted by the nematodes is sufficient to play a significant role in this reprograming. Previous studies quantified CKs secreted by *H. schachtii* and *M. incognita* which were $\sim 1.8X10^{-18}$ mol/larva^{-1}h^{-1} and 1.9×10^{-17} mol/larva^{-1}h^{-1} respectively, whereas the average concentration of CKs in host roots was $\sim 10^{-18}$ mol/nanoliter (nL) (De Meutter et al., 2003). With an average volume of a root cell ranging between 0.0005 and 0.5 nL, the additional CKs produced by the infective nematode are sufficient to influence the biological activity of the infected cells. Furthermore, CK levels in *HsIPT* knock-out *H. schachtii* showed a strong and significant decrease of one of the major active forms of CK in the syncytium; iP, with no change of total

CK concentration (Siddique et al., 2015). In addition, an infection assay in Arabidopsis by CK impaired *H. schachtii* showed no significant change of female size, but the number of females and the size of the syncytium were reduced in the host root which suggested that CKs are produced by *H. schachtii* exclusively for the successful parasitism.

The research project in the first part of the thesis aimed to study soil free-living *C. elegans*, and plant parasitic *H. glycines* through a comparative analysis to evaluate role of CKs in nematodes. While the earlier research on nematodes was limited to a single stage or mixed stage of nematode (Dimalla & Van Staden, 1977; De Meutter et al., 2003; Siddique et al., 2015), the present thesis investigated CKs at two different developmental stages of nematode (eggs and larvae). These two stages were selected based on the practical ability to isolate them, since SCN is an obligate parasite and must complete their life cycle inside the host plant. The aim of analyzing the egg stage was to determine CK profiles in the embryonic stage of *C. elegans* and *H. glycines*; therefore, similar CK profiles for *C. elegans* and *H. glycines* were expected for eggs stage as no parasitism occurs at this stage. Larva, in contrast, is the only infective stage of *H. glycines* and thus, it was considered as the most important stage to study CKs activity in *H. glycines*. In order to conduct the comparative analysis of these two species, *C. elegans* eggs and larvae stage were selected. Due to the lack of synchronized growth of *C. elegans* adult and presence of bacterial interference, the adult stage was not included in this study. Using HPLC positive electrospray ionization tandem mass spectrometry, a wide range of endogenous CKs were identified in *C. elegans* and *H. glycines* throughout the two developmental stages to test the hypothesis that *H. glycines* would have the more active CK profile (i.e. more biologically active forms and higher quantities of CKs) as compared to *C. elegans*. The endogenous hormone profiling revealed that *H. glycines* had significantly higher level of total CKs at both eggs and larvae stages than *C. elegans*; however, CKs in SCN were 93% (egg) and 97% (larva) in biologically precursor forms (i.e nucleotides; inactive in plant), whereas in *C. elegans*, 70% and 58% respectively, were bioactive forms (i.e free bases and ribosides). Therefore, the hypothesis was rejected and was suggested that CKs are present in *C. elegans* to perform an endogenous function in nematode physiology includes ageing. On the other hand, *H. glycines* may convert CKs from inactive to bioactive forms exclusively during parasitism. It has been demonstrated previously that exogenous application of CKs such as kinetin, para-topolin and meta-topolin prolong the lifespan and increase stress resistance, even though these compounds do not occur naturally in the organism (Kadlecová et al., 2018). CK biosynthesis gene tRNA-IPT has been identified in *C. elegans* and using that tRNA-IPT sequence from *C. elegans*, a single gene (*HsIPT*) ortholog encoding tRNA-IPT has been identified in the *H. schachtii* transcriptome and one putative full-length and two partial IPT protein sequences (*HgIPT*) have been identified in the draft genome of *H. glycines* (Siddique et al., 2015). No CK receptor gene has been identified in nematodes yet, therefore, no role for CKs in nematodes has been proposed. As mentioned above, *H. schachtii* produce CKs exclusively for plant parasitism, this finding on *H. schachtii* supports the idea that CKs in *H. glycines* may be converted from inactive (precursor forms, NTCKs) to bioactive (FBCKs) forms during infection of plant for successful parasitism. CKs synthesized by *H. schachtii* and *M. incognita* in the feeding sites were recognized by Arabidopsis histidine kinase (CKs receptors-AHK3 and AHK4) in Arabidopsis (Dowd et al., 2017; Siddique & Grundler, 2018). The same study detected that the expression of AHK4 and the negative regulator ARR15 were upregulated during the development of these special feeding sites. Additionally, the expression profile of the *HgIPT* gene throughout *H. glycines* development showed the highest expression during the early stage of infection and declines as the nematode proceeds to complete its life cycle (Elling et al., 2007). This supports the idea that CKs produced during the initial syncytium formation originate from nematodes. Interestingly, in this thesis, SCN showed higher content of inactive forms of CKs (NTCKs), suggesting that this pathogen might convert inactive NTCKs into FBCKs during syncytium formation only after the successful penetration in the host root. The second abundant group of CKs in *C. elegans* and *H. glycines* was 2MeSCKs, which has never been reported in nematodes previously. 2MeSCKs are widely identified CKs in plants and in many organisms beyond plants; however, their functions remain elusive. Recently it has been found that 2MeSCKs are associated with highly proliferating tissue, such as developing tumors in corn caused by the parasitic fungus *Ustilago maydis* (Morrison et al., 2015). Gall-forming phytopathogenic bacterium *Rhobdococcus fascians* exhibits a similar pattern of

2MeSCK involvement in continuous tissue proliferation by accumulating 2MeS-cZ in the infected tissue in the plant(Pertry et al., 2009). Based on the studies on gall forming bacterium and tumour forming fungus, it is anticipated that SCN synthesizes and subsequently capitalizes on the production of 2MeSCKs at the infective stage (larva) for their parasitism. The result of the current research supports this by showing 5-fold higher concentration of 2MeSCKs in SCN larva than their egg. On the other hand, 2MeSCKs concentration in *C. elegans* larva was detected 3-fold lower than their egg. This result support that 2MeSCKs might be primarily involved in nematode pathogenesis rather than in nematode physiology. But since no the role of 2MeSCKs has been confirmed, further research involving silencing key gene(s) that is(are) responsible for 2MeSCKs in nematode is required to observe the alteration of the endogenous hormone levels in association with the possible physiological changes.

Nematodes synthesize CKs via the tRNA degradation pathway

A discovery that nematodes produce CKs raised the question about which pathway they use to produce CKs. Any CKs synthesizing pathway in nematode was completely unknown until the tRNA-IPT sequence was identified in *C. elegans* (Sakakibara & Takei, 2002), *H. schachtii* and *H. glycines* (Siddique et al., 2015). These findings suggested the activation of tRNA degradation pathway in nematode. In this study, endogenous CK profiling revealed that iP and its conjugates were the predominant CK types, followed by 2MeSCKs and cZ type CKs in *C. elegans* and *H. glycines* egg and larva. While the tRNA-degradation pathway is widely accepted pathway for the cZ-type CK production, the question remains how nematode tRNA-IPT produces iP type CKs. The existing studies on fungi (Morrison et al., 2015) and bryophyte (*Physcomitrella patens)* (von Schwartzenberg et al., 2007) suggest that iP type CKs can be derived through tRNA degradation and also act as a possible source for the production of methylthiol derivatives. Therefore, the nematode tRNA-IPT enzyme may catalyze the production of a broader range of CKs. However, the trace levels of tZ, DZ and glucosides detected in *C. elegans* and *H. glycines* suggested that both species are also able to synthesize CKs via *de novo* pathway.

4.3 Role of CKs in soybean resistance to *H. glycines*

Continuing with the knowledge that CKs are important for parasitizing host in some nematode systems, such as the Arabidopsis-*H. schachtii* and Arabidopsis-*M. incognita* (Siddique et al., 2015; Dowd et al., 2017), in the second part of the thesis I tested the hypothesis that SCN resistance in soybean would alter CK dynamics both before and in response to SCN infection. I, therefore, profiled CKs in two SCN susceptible (Lee74 and Essex) and two resistant cultivars (Pion 22T69R and Pion 93Y05) at three soybean developmental stages (vegetative emergence, VE; vegetative stage 1, V1; and reproductive stage 1, R1). Consistent with the findings in *H. schachtii* and *M. incognita*, *H. glycines* infection stimulated all soybean root CK levels regardless of the cultivars resistance level. As essential growth-promoting signaling molecules in the plant kingdom, CKs play diverse roles in plant development, influencing many agriculturally important processes including growth, nutrient translocation and the response to biotic and abiotic stresses. Notably, CKs have also emerged as one of the virulence factors in plant-fungi, plant-insect and plant-nematode pathogenic interactions. As an example, *Ustilago maydis*, the causative agent of corn smut disease, is capable of producing CKs that play the key role in the *Ustilago maydis-Zea mays* pathosystem (Morrison et al., 2017). Herbivore insect, *Phyllonorycter blancardella* (leaf miner), produces and delivers CKs to their host plant to manipulate their nutritional microenvironment (Zhang et al., 2016, 2017). The role of CKs in the formation of giant cells (galls) induced by *M. incognita* in plant roots has been demonstrated previously (Lohar et al., 2004). Overall, it has been frequently identified that pathogen-originated CKs cause abnormal hypersensitivity of plants to CKs and also augment the sink activity of the infected regions (Walters & McRoberts, 2006; Dowd et al., 2017). Generally, nematode infection initiates complex changes in host plant gene expression (Gheysen & Fenoll, 2002). Genes that are induced in defense responses against the pathogen were upregulated in Arabidopsis infected by *H. schachtii*. The results of gene expression analysis in Arabidopsis revealed that 128 genes by *H. schachtii* and 12 genes by *H. glycines* (includes four stress/defense-regulated genes) were altered in expression during infection (Puthoff et al., 2003). Recent findings have shed light on a distinct role of plant-derived CKs in resistance against pathogen attack. This systematic resistance is orchestrated by endogenous CKs and salicylic acid (SA) signaling, where CKs and SA interact in plant immunity, but the underlying molecular mechanism is not clear yet (Choi et al., 2011).

To gain insight into the mechanism of SCN resistance, in the second part of the thesis, CK profiles of root segments of SCN susceptible (Lee74 and Essex) and resistant cultivars (Pion 22T69R and Pion 93Y05) were analyzed to test the hypothesis that SCN resistance in soybean would have altered CK dynamics both before, and in response to, SCN infection. The results showed that SCN infection caused a significant increase in soybean root CKs regardless of their resistance level. However, the increases were similar between the SCN susceptible and resistant soybean cultivars, both in control plants (SCN non-infected) and in response to SCN at all the analyzed developmental stages, except one of the resistant cultivars (Pion 93Y05) during V1 stage (figure 15 B), with significantly elevated levels of a plant bioactive CK, iP, in non-infected roots. This elevated level was comparable to the levels seen in the other three cultivars of SCN infected roots. This finding suggests that Pion 93Y05 has a resistance mechanism which was expressed through a consistently high level of iP. Additionally, the level of 2MeSZR at VE stage was significantly higher in the resistant cultivars than susceptible cultivars. These findings indicate a correlation between CK production and SCN resistance mechanism in soybean.

As mentioned earlier, the SCN resistant cultivars carry different genetic sources of resistance. Currently, there are three main sources for genetic resistance to SCN; PI 88788, PI 548402 (Peking), and PI 437654 (soybean lines that have the ability to resist nematode reproduction on their roots). Peking and PI 88788 are used as the main sources of SCN resistance in North American SCN resistant soybean cultivars (Concibidoet et.al., 2004). The comparison between these two types of SCN resistance sources in soybean was reviewed by Mitchum (2016). Penetration of the nematode into roots of resistant soybeans is not impacted in SCN resistant genotypes, but the fecundity of nematodes is suppressed in resistant cultivars (Li et al., 2004) by means of collapsing syncytium. Peking or cultivars developed from it, show rapid syncytium degeneration, whereas PI 88788 or cultivars developed from this source, exhibit a slower degeneration of the syncytium. Early histological studies suggested this type of degrading syncytium in resistant cultivars is an active defense response, or resistance response (Martin & McCarthy, 1990). Despite the extensive histological studies documenting the cellular changes associated with degenerating syncytium in the host plant, very little is known about the molecular mechanisms of SCN resistance. Genetic analyses showed that the resistance to SCN is controlled by multiple genes. There are several genes identified that are responsible for SCN resistance. There are three recessive genes *rhg1, rhg2,* and *rhg3* (Caldwell et al., 1960) and two dominant genes *Rhg4* and *Rhg5* (Rao- Arelli et al., 1992, 1994), where the major resistance gene in most SCN resistance sources is *rhg1* (Brucker et. al., 2005), while *Rhg4* is required for full resistance to certain SCN populations from some but not all resistant sources (Brucker et al., 2005). Genetic mapping studies have established that PI 88788-type resistance requires *rhg1*, whereas Peking-type resistance has the additional requirement for *rhg4* to confer resistance to SCN (Concibido et al., 2004; Meksemet al. 2001). The two SCN resistant cultivars used in this thesis, Pion 22T69R and Pion 93Y05, have the source of SCN resistance from Peking and PI 88788 respectively, and it is already known that their genetic requirement for resistance to SCN is different. So, based on this knowledge, it is confirmed that Pion 93Y05 is comparatively less resistant to SCN than the other resistant cultivar, Pion 22T69R.

There is no research primarily conducted on CK involvement in SCN resistance mechanism in soybean. However, the application of exogenous CKs to *M. incognita* resistant tomato cultivars resulted in more nematode growth, less necrosis of the feeding cells, but overall, resistance was not broken (Dropkin et al., 1969). It can reasonably be expected that once the nematode starts feeding and its metabolism intensifies, the accumulation of CKs may increase concomitantly and trigger a change in the nematode, together with the CK production that originates from the plant. Thus, the high level of CKs can favor parasitic nematode growth and development by establishing successful parasitism. Moreover, it has been found that CK deficiency reduces the virulence of *H. schachtii* (Siddique et al., 2015). To date, no similar studies have been conducted on the SCN or soybean-SCN system. Therefore, exogenous application of CKs in SCN resistant soybean cultivars may assist in determining how or if CKs are involved in the underlying molecular mechanism of SCN resistance in soybean.

5 Conclusions

The aim of this study was to gain insight into the role of CKs in nematodes and to investigate the influence of plant parasitic nematode originated CKs in pathogenesis. In this thesis, the comprehensive study on endogenous CKs in free-living *C. elegans* and plant parasitic *H. glycines* revealed that CKs may regulate endogenous function in *C. elegans* physiology and on the other side, *H. glycines* produces CKs primarily for successful parasitism. The most novel finding in this study was the presence of 2MeSCKs in both species of nematodes. The importance of 2MeSCKs in *H. glycines* pathogenesis was indicated by the level detected in *H. glycines* larva.

In the second part of the thesis, four soybean cultivars differing in SCN resistance level were examined to demonstrate a potential role of CKs in soybean resistance to SCN. The results showed SCN caused a significant increase of soybean roots CKs regardless of resistance level. Furthermore, no statistically significant difference was observed among the tested cultivars except one SCN resistant cultivar Pion 93Y05. A highly elevated level of bioactive iP in non-infected root was detected in Pion 93Y05 which was comparable to the levels seen in the other three cultivars of SCN infected roots. This finding indicated that Pion 93Y05 has a resistance mechanism which was exhibited through a consistently high level of iP. In summary, the research project in this thesis presented metabolic evidence of the production of a broad range of CKs by *C. elegans* and *H. glycines* and clarified nematode CK biosynthesis pathway, shed light on *H. glycines* ability to stimulate soybean root CK production and finally demonstrated a potential role of CKs in soybean resistance to SCN.

6 Future directions

The set of experiments presented in this thesis creates a preliminary platform for future studies on CKs in the nematode species and on SCN resistance mechanism associated with CK production. Clarifying further details of the activity of nematode tRNA-IPT gene such as *gro-1*, *HgIPT* may provide exciting information on the physiological role of CKs in *C. elegans* and *H. glycines*. One way to accomplish this would be silencing tRNA-IPT gene in nematode and determining whether the resulting pathogenesis, CK profiles and associated physiological traits are altered. In reference to the second part of the thesis, soybean response to SCN infection confirmed stimulation of nematode infection in soybean CK production. However, the correlation between CK production and SCN resistance in host plant needs to be further investigated in other resistant cultivars containing various sources of SCN resistance which can be used to develop a potential biological marker for SCN resistance in soybean.

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8 Appendix

8.1 Soybean plant cultivation and SCN infection

Soybean plants were grown in the greenhouse for 40 days. Greenhouse conditions were 25^0C with 16 hours light (300 watts/m^2 intensity), water temperature 27^0C. Each plant was grown in a plastic cone having a hole at the bottom. Cones were filled with sterile sandy loam soil (75% sand) and a hole was made in the soil to place the seeds. 4000 SCN eggs were used as inoculum (1.5 – 2.0 mL of egg suspension) for the infection. To prepare SCN inoculum, previously collected cysts were crushed to extract eggs following the SCN egg extraction procedure. The soybean roots were collected at three developmental stages; vegetative emergence (VE) (5 DAS without SCN infection), vegetative stage (V1) (14 DAS) and reproductive stage (R1) (40 DAS) for CK analysis.

8.2 Collection of SCN cyst from soybean root

To collect cysts from the roots, the plant stem was cut at the soil line. The whole root system was gently removed from the soil and placed into a two-gallon bucket containing water to remove the extra soil. The plant root was gently rubbed to dislodge excess soil and placed on a nested sieve; 30 mesh (600 µm in diameter pores) sieve on top of 60 mesh (250 µm in diameter pores). The whole root system was washed with high powered hose water thoroughly to detach all the cyst. All the roots were visually inspected not to miss any cysts. The 30 mesh sieve was removed with all the debris and the 60 mesh sieve with the cysts was slightly tilted and carefully rinsed everything trapped on the screen. The cysts were collected at the edge of the sieve by gently washing the inside with running water. The cysts were rapidly poured into a sterile 500 ml plastic beaker. The water containing the cysts was poured into a 1 L glass cylinder. After 20 to 30 minutes, cysts sedimented to the bottom of the cylinder. Water decanted as much as possible from the top of the cylinder. Fresh sucrose solution (454 grams/L) was added to the cysts suspension in the cylinder. Fresh sugar solution was used to avoid fungal growth. The sucrose solution was added to a final volume of 500 ml into the cylinder. A piece of parafilm was placed over the top of the cylinder and turned upside down twice to mix the solution. In 15 minutes cysts were floated on the top of the sugar solution separating them from most of the soil particles and root debris. To clean the cysts, they were decanted into a 1 L glass beaker. The cylinder was rotated while pouring to recover the cysts stuck on the walls of the cylinder. The solution from the beaker was poured into a 3-inch diameter 60 mesh sieve and rinsed well to remove the sucrose solution. The content of the sieve was collected in a 250 ml beaker.

Before discarding soil and the roots waste, all materials were autoclaved. The wastewater bucket was treated with 10% bleach solution to kill any remaining nematodes in it.

8.3 SCN eggs extraction from cyst

The females (cysts) were grounded, using a rubber stopper, on the surface of a 100 mesh sieve (150 µm in diameter pores) nested over a 200 (75 µm in diameter pores) and a 500 (25 µm in diameter pores) mesh sieves. Liberated eggs and fine debris were collected from the 500 mesh sieve to a 50 mL tube and centrifuged at 2000 rpm for 4 minutes. Excess water was discarded, and the eggs pellet was re-suspended with sterile water and carefully layered on the surface of a 30-40ml sugar water mixture (45.5%) in the same tube. The mixture was then centrifuged again at 2000 RPM for 4 minutes. The resulting band of eggs, a white layer at the meniscus between the water and sugar solution, was then pipetted off into the 500 mesh sieve and rinsed with distilled water. Extracted eggs were sterilized by placing the 500-mesh sieve with eggs, in an autoclaved 4 cm deep plastic tray with a bleach solution (0.4% sodium hypochlorite solution) for 1.5 minutes and rotated the sieve. The eggs were rinsed with 1 L sterile water after bleaching and transferred to a sterile bottle.

Table 8.1: Raw data of CKs content in nematodes at their two developmental stages; egg and larva. Concentration is in pmol nematode^{-1}. ND represents CKs that were not detected.

Species	Stage	FB				RB				NT				MES			
		***trans*-Z**	***Cis*-Z**	**DZ**	**iP**	***trans*-ZR**	***Cis*-ZR**	**DZR**	**iPR**	***trans*-ZNT**	***Cis*-ZNT**	**DZNT**	**iPNT**	**2-MeSZ**	**2-MeSiP**	**2-MeSZR**	**2-MeSiPA**
SCN	Egg	5.79E-07	1.74E-06	ND	1.42E-06	ND	5.76E-08	3.03E-06	3.39E-06	ND	1.57E-07	1.57E-07	1.36E-04	4.30E-06	5.71E-06	7.07E-07	7.72E-07
	Egg	4.68E-07	1.83E-06	ND	1.14E-06	ND	ND	2.24E-06	3.53E-06	1.47E-07	ND	5.35E-08	1.30E-04	2.57E-06	2.60E-06	1.73E-07	5.19E-07
	Egg	ND	4.68E-06	ND	1.67E-06	ND	ND	4.42E-06	7.89E-06	ND	ND	2.22E-07	2.45E-04	5.12E-06	3.35E-06	3.07E-07	7.05E-07
	Larva	ND	3.37E-06	ND	2.37E-06	ND	1.78E-07	2.23E-07	2.66E-07	ND	ND	ND	1.26E-04	1.61E-05	2.11E-05	ND	8.62E-07
	Larva	ND	2.47E-06	ND	ND	ND	ND	2.50E-07	2.94E-07	ND	ND	ND	7.98E-05	1.28E-05	2.36E-05	6.71E-07	ND
	Larva	1.14E-06	7.11E-07	ND	ND	ND	ND	9.97E-07	2.87E-07	ND	ND	ND	1.21E-04	6.34E-06	1.76E-05	ND	ND
C.elegans	Egg	ND	ND	ND	3.40E-05	4.98E-07	4.98E-07	1.41E-07	4.45E-07	1.58E-07	9.30E-07	ND	4.32E-06	1.64E-06	1.47E-06	1.64E-07	1.37E-07
	Egg	2.24E-06	2.81E-06	3.72E-07	1.11E-05	3.92E-07	1.82E-06	4.54E-07	1.35E-06	6.43E-08	6.04E-07	ND	4.01E-06	9.18E-06	3.42E-06	3.93E-07	5.60E-07
	Egg	3.32E-06	3.01E-06	3.82E-07	3.76E-05	5.71E-07	1.02E-05	4.53E-07	2.42E-06	3.87E-07	1.88E-06	ND	3.84E-06	1.09E-05	3.81E-06	4.32E-07	6.89E-07
	Larva	ND	ND	ND	1.76E-06	ND	1.71E-07	2.38E-07	1.24E-06	9.71E-08	4.00E-07	ND	1.54E-05	5.38E-07	1.01E-06	7.64E-08	1.37E-07
	Larva	ND	1.33E-06	3.74E-07	5.53E-06	9.48E-08	1.27E-07	2.76E-07	5.90E-07	ND	4.55E-07	ND	1.13E-05	5.92E-06	2.16E-06	1.88E-07	1.70E-07
	Larva	8.54E-07	ND	3.45E-07	8.25E-05	1.60E-07	2.92E-07	3.21E-07	1.11E-06	1.67E-07	4.13E-07	ND	2.65E-05	2.89E-06	8.71E-07	1.03E-07	2.05E-07

Table 8.2: Average of FBCKs in nematodes at their two developmental stages; egg and larva. Concentration is in pmol nematode^{-1}. NS = not significant, *P* value < 0.05. ND represents CKs that were not detected.

Species	**Stages**	**tZ**	**cZ**	**DZ**	**iP**
SCN	Eggs	3.48E-07 ± 1.45E-07 **NS**	2.75E-06 ± 7.90E-07 **NS**	ND	1.41E-06 ± 1.24E-07 **NS**
SCN	Juvenile	3.79E-07 ± 3.09E-07	2.18E-06 ± 6.38E-07	ND	7.91E-07 ± 6.46E-07
C. elegans	Egg	1.85E-06 ± 7.98E-07	1.94E-06 ± 7.94E-07	2.51E-07 ± 1.03E-07 **NS**	2.76E-05 ± 6.78E-06
C. elegans	Larva	2.85E-07 ± 2.32E-07	4.42E-07 ± 3.61E-07	2.40E-07 ± 9.82E-08	2.99E-05 ± 2.15E-05

Table 8.3: Average of RBCKs in nematodes at their two developmental stages; egg and larva. Concentration is in pmol nematode^{-1}. P value < 0.05. ND represents CKs that were not detected.

Species	Stages	tZR	cZR	DZR	iPR
SCN	Eggs	ND	1.9E-08 ± 2E-08 **NS**	3.2E-06 ± 5.2E-07 **a**	4.9E-06 ± 1.2E-06 **a**
SCN	Juvenile	ND	5.9E-08 ± 5E-08	4.9E-07 ± 2.1E-07 **b**	2.8E-07 ± 6.7E-09 **b**
C. elegans	Egg	5E-07 ± 4E-08 **a**	4.2E-06 ± 2E-06	3.5E-07 ± 8.5E-08 **b**	1.4E-06 ± 4.7E-07 **b**
C. elegans	Larva	8E-08 ± 4E-08 **b**	2E-07 ± 4E-08	2.8E-07 ± 1.9E-08 **b**	9.8E-07 ± 1.6E-07 **b**

Table 8.4: Average of NTCK groups in nematodes at their two developmental stages; egg and larva. Concentration is in pmol nematode^{-1}. P value < 0.05. ND represents CKs that were not detected.

Species	Stages	tZNT	cZNT	DZNT	iPNT
SCN	Eggs	5E-08 ± 4E-08 **NS**	5.2E-08 ± 4E-08 **b**	1.4E-07 ± 4E-08 **a**	1.70E-04 ± 3.1E-05 **a**
SCN	Juvenile	ND	ND	ND	1.09E-04 ± 1.2E-05 **a**
C. elegans	Egg	2E-07 ± 8E-08	1.1E-06 ± 3E-07 **a**	ND	4.1E-06 ± 1.2E-07 **b**
C. elegans	Larva	9E-08 ± 4E-08	4.2E-07 ± 1E-08 **b**	ND	1.8E-05 ± 3.7E-06 **b**

Table 8.5: Average of 2MeSCK groups in nematodes at their two developmental stages; egg and larva. Concentration is in pmol nematode^{-1}. P value < 0.05.

Species	Stages	2MeSZ	2MeSiP	2MeSZR	2MeSiPA
SCN	Eggs	4E-06 ± 6E-07 **b**	3.9E-06 ± 8E-07 **b**	4E-07 ± 1.3E-07 **NS**	6.7E-07 ± 6.2E-08 **NS**
SCN	Juvenile	1E-05 ± 2E-06 **a**	2.1E-05 ± 1E-06 **a**	2.2E-07 ± 1.8E-07	2.9E-07 ± 2.3E-07
C. elegans	Egg	7E-06 ± 2E-06 **ab**	2.9E-06 ± 6E-07 **b**	3.3E-07 ± 6.8E-08	4.6E-07 ± 1.4E-07
C. elegans	Larva	3E-06 ± 1E-06 **b**	1.3E-06 ± 3E-07 **b**	1.2E-07 ± 2.8E-08	1.7E-07 ± 1.6E-08

Table 8.6: Average of FBs, RBs, NTs, 2MeSCKs and glucosides in nematodes at their two developmental stages; egg and larva. Concentration is in pmol nematode^{-1}. P value < 0.05.

Species	Stages	FB	RB	NT	2MeS	Glucosides
SCN	Eggs	5E-06 ± 8E-07 **NS**	8.2E-06 ± 2E-06 **a**	1.70E-04 ± 3E-05 **a**	8.9E-06 ± 1.3E-06 **b**	5E-07 ± 2E-07 **a**
SCN	Juvenile	3E-06 ± 1E-06	8.3E-07 ± 2E-07 **b**	1.09E-04 ± 1.2E-05 **a**	3.3E-05 ± 3.7E-06 **a**	4E-08 ± 3E-08 **b**
C. elegans	Egg	3E-05 ± 7E-06	6.4E-06 ± 3E-06 **ab**	5.4E-06 ± 3.4E-07 **b**	1.1E-05 ± 3.1E-06 **b**	6E-08 ± 4E-08 **b**
C. elegans	Larva	3E-05 ± 2E-05	1.5E-06 ± 2E-07 **ab**	1.8E-05 ± 3.7E-06 **b**	4.8E-06 ± 1.6E-06 **b**	2E-08 ± 1E-08 **b**

Table 8.7: Raw data of soybean at VE stage. Concentration is in pmol g^{-1}DWt. ND represents CKs that were not detected.

	FB				RB				NT				MES			
Cultivars-Reps	**tZ**	**cisZ**	**DZ**	**iP**	**tZR**	**cisZR**	**DZR**	**iPR**	**tZNT**	**cisZNT**	**DZNT**	**iPNT**	**MeSZ**	**MeSiP**	**MeSZR**	**MeSiPA**
Lee74-1	ND	119.09	14.81	58.07	95.92	45.64	24.40	81.05	8.53	49.58	0.26	52.35	30.07	24.14	6.32	0.87
Lee74-2	69.07	50.41	81.82	ND	17.15	50.22	7.64	33.34	3.77	35.52	0.13	67.65	232.08	100.88	0.49	ND
Lee74-3	25.71	13.67	12.69	90.28	30.44	39.16	15.31	26.65	5.23	32.13	0.11	35.47	29.46	11.64	12.52	12.30
Essex-1	27.34	13.71	14.30	84.49	54.45	111.95	14.64	125.81	5.10	44.44	ND	28.04	144.20	51.68	3.31	54.55
Essex-2	11.80	9.29	5.49	45.54	8.96	34.70	3.57	33.15	6.59	59.75	ND	157.69	445.33	231.60	0.06	374.71
Essex-3	40.65	28.62	28.27	105.85	29.35	150.55	7.74	44.03	5.74	68.21	ND	201.92	40.17	13.25	11.24	29.57
PION P22T69R-1	ND	ND	6.53	82.65	22.19	57.16	6.92	60.13	6.00	44.49	3.67	228.27	68.11	27.86	5.89	24.91
PION P22T69R-2	18.49	20.71	13.35	78.22	48.71	81.55	16.24	73.78	2.40	35.39	ND	ND	67.07	35.93	2.66	30.84
PION P22T69R-3	19.00	12.59	11.30	59.95	8.04	7.69	4.12	6.94	10.21	51.67	ND	98.41	7.70	6.30	344.35	0.30
PION 93Y05-1	34.09	30.69	12.31	77.92	13.64	34.55	8.32	29.27	3.38	32.91	0.23	27.36	30.44	14.17	83.74	17.67
PION 93Y05-2	30.99	36.01	15.11	122.32	43.79	101.45	12.26	67.27	2.01	39.99	ND	17.13	155.71	61.97	703.17	110.29
PION 93Y05-3	7.03	7.22	4.67	42.65	70.77	48.61	18.46	66.15	6.11	59.44	ND	67.41	25.70	7.57	871.99	12.45

Table 8.8: Average of different FBCKs in soybean cultivars at VE stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05. ND represents CKs that were not detected.

Name	SCN susceptibility	Treatment	tZ			cZ			DZ			iP		
Lee74	SCN susceptible	SCN non-infected	31.59	±	16.46 **NS**	61.06	±	25.22 **NS**	36.44	±	18.53 **NS**	49.45	±	21.57 **NS**
Essex	SCN susceptible	SCN non-infected	26.60	±	6.81	17.20	±	4.77	16.02	±	5.41	78.63	±	14.41
PION P22T69R	SCN resistant	SCN non-infected	12.50	±	5.10	11.10	±	4.92	10.39	±	1.65	73.61	±	5.67
PION 93Y05	SCN resistant	SCN non-infected	24.04	±	6.98	24.64	±	7.22	10.70	±	2.55	80.96	±	18.82

Table 8.9: Average of different RBCKs in soybean cultivars at VE stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05. ND represents CKs that were not detected.

Name	SCN susceptibility	Treatment	tZR			cZR			DZR			iPR		
Lee74	SCN susceptible	SCN non-infected	47.84	±	19.88 NS	45.01	±	2.62 NS	15.78	±	3.95 NS	47.01	±	13.98 NS
Essex	SCN susceptible	SCN non-infected	30.92	±	10.74	99.07	±	27.81	8.65	±	2.64	67.66	±	23.88
PION P22T69R	SCN resistant	SCN non-infected	26.31	±	9.73	48.80	±	17.74	9.09	±	2.99	46.95	±	16.65
PION 93Y05	SCN resistant	SCN non-infected	42.74	±	13.47	61.54	±	16.63	13.01	±	2.41	54.23	±	10.19

Table 8.10: Average of different NTCKs in soybean cultivars at VE stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05. ND represents CKs that were not detected.

Name	SCN susceptibility	Treatment	tZNT			cZNT			DZNT			iPNT		
Lee74	SCN susceptible	SCN non-infected	5.84	±	1.15 NS	39.07	±	4.36 NS	0.17	±	0.04 NS	51.82	±	7.59 NS
Essex	SCN susceptible	SCN non-infected	5.81	±	0.35	57.47	±	5.68	ND			129.22	±	42.60
PION P22T69R	SCN resistant	SCN non-infected	6.20	±	1.84	43.85	±	3.85	1.22	±	1.00	108.89	±	53.97
PION 93Y05	SCN resistant	SCN non-infected	3.83	±	0.99	44.11	±	6.48	0.08	±	0.06	37.30	±	12.53

Table 8.11: Average of different 2MeSCKs in soybean cultivars at VE stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Name	SCN susceptibility	Treatment	2MeSZ	2MeSiP	2MeSZR	2MeSiPR
Lee74	SCN susceptible	SCN non-infected	97.20 ± 55.06 NS	45.56 ± 22.78 NS	6.44 ± 2.84 b	4.39 ± 3.23 NS
Essex	SCN susceptible	SCN non-infected	209.90 ± 99.19	98.84 ± 54.95	4.87 ± 2.71 b	152.95 ± 90.73
PION P22T69R	SCN resistant	SCN non-infected	47.63 ± 16.30	23.36 ± 7.22	117.63 ± 92.56 b	18.68 ± 7.63
PION 93Y05	SCN resistant	SCN non-infected	70.62 ± 34.76	27.90 ± 14.00	552.96 ± 195.65 a	46.80 ± 25.95

Table 8.12: Average of FBs, RBs, NTs, 2MeSCKs and glucosides in soybean cultivars at VE stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Name	SCN susceptibility	Treatment	FB	RB	NT	2MeS	Glucosides
Lee74	SCN susceptible	SCN non-infected	178.54 ± 14.94 NS	155.64 ± 37.31 NS	96.91 ± 9.82 NS	153.59 ± 73.43 NS	8.16 ± 3.14 NS
Essex	SCN susceptible	SCN non-infected	138.45 ± 30.94	206.29 ± 54.38	192.49 ± 48.48	466.56 ± 241.82	5.96 ± 0.93
PION P22T69R	SCN resistant	SCN non-infected	107.60 ± 10.00	131.16 ± 46.03	160.17 ± 57.66	207.31 ± 61.83	3.16 ± 1.02
PION 93Y05	SCN resistant	SCN non-infected	140.34 ± 34.20	171.51 ± 35.34	85.32 ± 19.48	698.29 ± 227.04	5.62 ± 2.20

Table 8.13: Raw data of soybean at V1 stage. Concentration is in pmol g^{-1}DWt . ND represents CKs that were not detected.

			FB				RB				NT				MES			
Cultivars-Reps	**Treatment**	**Susceptibility**	**tZ**	**cisZ**	**DZ**	**iP**	**tZR**	**cisZR**	**DZR**	**iPR**	**tZNT**	**cisZNT**	**DZNT**	**iPNT**	**MeSZ**	**MeSiP**	**MeSZR**	**MeSiPA**
Lee74-1	Control	SCN susceptible	29	192	51	4650	42	20	11	36	24	28	3	261	13	11	3	ND
Lee74-2	Control	SCN susceptible	145	124	690	23689	17	50	8	33	10	53	ND	327	81	101	2	ND
Lee74-3	Control	SCN susceptible	118	49	330	25444	26	33	13	23	24	33	2	286	25	10	7	10
Lee74-4	Control	SCN susceptible	49	49	209	10452	24	49	6	55	14	30	1	54	63	23	3	24
Lee74-1	SCN infected	SCN susceptible	31	143	13	58245	140	56	16	94	103	34	5	247	33	66	6	1
Lee74-3	SCN infected	SCN susceptible	93	56	136	41415	212	52	38	169	173	55	15	520	63	213	6	50
Lee74-4	SCN infected	SCN susceptible	56	36	64	61418	119	47	25	106	124	57	7	356	27	14	4	15
Essex-1	Control	SCN susceptible	188	172	541	8340	10	37	4	36	5	20	ND	45	60	6	2	99
Essex-2	Control	SCN susceptible	202	143	484	7907	21	110	6	32	29	100	ND	552	29	10	6	22
Essex-3	Control	SCN susceptible	712	975	2004	10177	14	36	4	38	14	36	ND	202	43	18	3	16
Essex-4	Control	SCN susceptible	570	911	1566	7522	32	54	11	49	13	31	ND	144	45	24	4	21
Essex-1	SCN infected	SCN susceptible	64	41	92	99010	199	114	16	158	86	45	3	357	55	29	4	52
Essex-2	SCN infected	SCN susceptible	38	25	42	43409	101	38	15	88	76	35	3	237	29	14	4	15
Essex-3	SCN infected	SCN susceptible	56	36	60	62435	114	54	13	108	92	35	3	285	54	62	4	74
Essex-4	SCN infected	SCN susceptible	61	47	104	89316	146	53	25	179	82	42	3	349	55	25	6	18
PION P22T69R-1	Control	SCN resistant	ND	1389	12	4428	20	19	10	17	24	43	1	91	19	15	6	1
PION P22T69R-2	Control	SCN resistant	32	12	56	3519	13	32	8	27	10	46	ND	90	28	13	6	16
PION P22T69R-3	Control	SCN resistant	25	12	21	7193	20	47	6	31	5	24	ND	80	72	29	3	51
PION P22T69R-4	Control	SCN resistant	25	26	36	11733	63	43	16	59	22	60	2	155	23	7	4	11
PION P22T69R-1	SCN infected	SCN resistant	59	375	15	58864	46	41	9	113	35	42	2	321	21	23	7	1
PION P22T69R-2	SCN infected	SCN resistant	63	39	64	75006	149	52	25	135	13	26	1	156	57	22	5	33
PION P22T69R-3	SCN infected	SCN resistant	35	27	40	52630	18	26	11	46	36	43	2	395	40	23	6	36
PION P22T69R-4	SCN infected	SCN resistant	71	56	85	101678	82	82	17	229	15	84	1	448	56	23	9	48
Pion93Y05-1	Control	SCN resistant	30	21	22	2556	25	33	9	57	12	27	ND	86	55	18	3	29
Pion93Y05-2	Control	SCN resistant	59	39	205	160826	14	37	5	21	12	45	ND	127	54	26	4	31
Pion93Y05-3	Control	SCN resistant	56	70	249	54715	54	44	8	48	12	19	ND	51	48	22	2	21
Pion93Y05-4	Control	SCN resistant	42	27	103	68451	38	38	10	18	11	33	ND	40	56	23	4	29
Pion93Y05-1	SCN infected	SCN resistant	40	38	45	47402	27	76	10	111	34	36	1	229	71	10	7	18
Pion93Y05-2	SCN infected	SCN resistant	75	67	95	87779	102	108	14	224	37	51	2	409	94	23	13	46
Pion93Y05-3	SCN infected	SCN resistant	68	46	61	62410	49	80	15	101	85	87	4	490	64	12	8	25
Pion93Y05-4	SCN infected	SCN resistant	58	49	66	58588	106	145	16	181	113	73	4	486	51	18	11	37

Table 8.14: Average of different FBCKs in soybean cultivars at V1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Cultivars	SCN susceptibility	Treatment	tZ	cZ	DZ	iP
Lee74	SCN susceptible	SCN non-infected	84.96 ± 23.79 **b**	103.48 ± 29.69 **ab**	319.89 ± 117.59 **b**	16058.67 ± 4386.70 **b**
Essex	SCN susceptible	SCN non-infected	417.56 ± 114.33 **a**	549.99 ± 196.74 **a**	1148.89 ± 327.64 **a**	8486.42 ± 508.94 **b**
PION P22T69R	SCN resistant	SCN non-infected	20.62 ± 6.08 **b**	359.83 ± 296.98 **ab**	31.15 ± 8.32 **b**	6718.09 ± 1597.98 **b**
PION 93Y05	SCN resistant	SCN non-infected	46.67 ± 5.75 **b**	39.11 ± 9.40 **b**	145.05 ± 44.25 **b**	71636.88 ± 28530.07 **a**
Lee74	SCN susceptible	SCN infected	59.70 ± 14.76 **b**	78.67 ± 26.85 **b**	70.71 ± 29.08 **b**	53692.69 ± 5067.91 **ab**
Essex	SCN susceptible	SCN infected	54.92 ± 5.04 **b**	37.48 ± 4.01 **b**	74.61 ± 12.42 **b**	73542.45 ± 10979.47 **a**
PION P22T69R	SCN resistant	SCN infected	56.78 ± 6.63 **b**	124.21 ± 72.67 **ab**	50.86 ± 13.09 **b**	72044.75 ± 9478.85 **a**
PION 93Y05	SCN resistant	SCN infected	60.00 ± 6.56 **b**	49.93 ± 5.25 **b**	66.85 ± 9.02 **b**	64044.90 ± 7385.48 **a**

Table 8.15: Average of different RBCKs in soybean cultivars at V1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Cultivars	SCN susceptibility	Treatment	tZR	cZR	DZR	iPR
Lee74	SCN susceptible	SCN non-infected	27.27 ± 4.57 **b**	38.20 ± 6.21 **b**	9.46 ± 1.30 **ab**	36.71 ± 5.86 **b**
Essex	SCN susceptible	SCN non-infected	19.44 ± 4.31 **b**	59.61 ± 15.02 **b**	6.19 ± 1.38 **b**	38.88 ± 3.17 **b**
PION P22T69R	SCN resistant	SCN non-infected	28.83 ± 9.94 **b**	35.15 ± 5.46 **b**	9.95 ± 2.02 **ab**	33.43 ± 7.75 **b**
PION 93Y05	SCN resistant	SCN non-infected	32.68 ± 7.46 **b**	37.99 ± 2.07 **b**	8.05 ± 0.87 **ab**	35.96 ± 8.43 **b**
Lee74	SCN susceptible	SCN infected	157.16 ± 23.04 **a**	51.52 ± 2.20 **b**	26.18 ± 5.30 **a**	122.97 ± 18.93 **ab**
Essex	SCN susceptible	SCN infected	139.93 ± 18.97 **a**	64.82 ± 14.42 **b**	17.19 ± 2.38 **ab**	133.48 ± 18.42 **a**
PION P22T69R	SCN resistant	SCN infected	73.77 ± 24.61 **ab**	50.47 ± 10.20 **b**	15.44 ± 3.18 **ab**	130.67 ± 32.71 **a**
PION 93Y05	SCN resistant	SCN infected	70.93 ± 17.04 **ab**	102.16 ± 13.81 **a**	13.68 ± 1.29 **ab**	154.18 ± 25.32 **a**

Table 8.16: Average of different NTCKs in soybean cultivars at V1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Cultivars	SCN susceptibility	Treatment	tZNT	cZNT	DZNT	iPNT
Lee74	SCN susceptible	SCN non-infected	18.03 ± 3.20 c	36.04 ± 4.96 NS	1.21 ± 0.50 bc	232.18 ± 52.64 ab
Essex	SCN susceptible	SCN non-infected	15.33 ± 4.24 c	46.66 ± 15.78	0.00 ± 0.00 c	235.92 ± 95.55 ab
PION P22T69R	SCN resistant	SCN non-infected	15.42 ± 4.00 c	43.37 ± 6.40	0.92 ± 0.48 bc	103.76 ± 14.88 b
PION 93Y05	SCN resistant	SCN non-infected	11.80 ± 0.26 c	31.03 ± 4.75	0.00 ± 0.00 c	76.07 ± 17.09 b
Lee74	SCN susceptible	SCN infected	124.54 ± 15.00 a	43.99 ± 6.20	8.07 ± 1.91 a	345.50 ± 54.75 a
Essex	SCN susceptible	SCN infected	83.85 ± 2.86 b	39.21 ± 2.06	3.26 ± 0.07 b	307.32 ± 24.58 a
PION P22T69R	SCN resistant	SCN infected	24.63 ± 5.35 c	48.77 ± 10.85	1.25 ± 0.23 bc	330.20 ± 54.95 a
PION 93Y05	SCN resistant	SCN infected	67.23 ± 16.76 b	61.98 ± 9.79	2.77 ± 0.51 bc	403.51 ± 52.96 a

Table 8.17: Average of different 2MeSCKs in soybean cultivars at V1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Name	SCN susceptibility	Treatment	2MeSZ	2MeSiP	2MeSZR	2MeSiPR
Lee74	SCN susceptible	SCN non-infected	45.62 ± 13.75 ab	36.02 ± 18.89 NS	3.67 ± 0.93 bc	8.70 ± 4.88 NS
Essex	SCN susceptible	SCN non-infected	44.34 ± 5.38 ab	14.41 ± 3.46	3.61 ± 0.78 c	39.19 ± 17.20
PION P22T69R	SCN resistant	SCN non-infected	35.38 ± 10.63 b	15.94 ± 3.97	4.86 ± 0.59 bc	19.72 ± 9.40
PION 93Y05	SCN resistant	SCN non-infected	53.58 ± 1.54 ab	22.10 ± 1.36	3.45 ± 0.44 c	27.75 ± 1.88
Lee74	SCN susceptible	SCN infected	40.84 ± 9.22 b	97.72 ± 48.55	5.46 ± 0.51 bc	21.97 ± 11.95
Essex	SCN susceptible	SCN infected	48.49 ± 5.59 ab	32.61 ± 8.95	4.39 ± 0.40 bc	39.69 ± 12.32
PION P22T69R	SCN resistant	SCN infected	43.42 ± 7.15 ab	22.73 ± 0.13	6.87 ± 0.73 ab	29.46 ± 8.71
PION 93Y05	SCN resistant	SCN infected	70.01 ± 7.65 a	15.63 ± 2.41	9.44 ± 1.29 a	31.59 ± 5.38

Table 8.18: Average of FBs, RBs, NTs, 2MeSCKs and glucosides in soybean cultivars at V1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Name	SCN susceptibility	Treatment	FB	RB	NT	2MeS	Glucs
Lee74	SCN susceptible	SCN non-infected	16,567.00 ± 4,489.53 **b**	111.63 ± 7.16 **b**	287.46 ± 55.83 **bc**	94.02 ± 30.18**NS**	6.26 ± 1.23 **b**
Essex	SCN susceptible	SCN non-infected	10,602.87 ± 1,000.07 **b**	124.12 ± 17.54 **b**	297.92 ± 115.42**abc**	101.55 ± 19.35	4.83 ± 0.72 **b**
PION P22T69R	SCN resistant	SCN non-infected	7,129.69 ± 1,501.04 **b**	107.37 ± 22.39 **b**	163.48 ± 23.76 **c**	75.90 ± 23.04	2.96 ± 0.78 **b**
PION 93Y05	SCN resistant	SCN non-infected	71,867.71 ± 28,563.07 **a**	114.69 ± 14.17 **b**	118.89 ± 20.83 **c**	106.87 ± 4.18	7.42 ± 0.67 **b**
Lee74	SCN susceptible	SCN infected	53,901.76 ± 4,361.52**ab**	357.82 ± 39.88 **ab**	522.10 ± 76.53 **ab**	165.99 ± 59.63	8.97 ± 3.83 **ab**
Essex	SCN susceptible	SCN infected	73,709.46 ± 10,999.48 **a**	355.43 ± 47.91 **a**	433.65 ± 27.46 **ab**	125.19 ± 24.31	15.93 ± 4.55 **a**
PION P22T69R	SCN resistant	SCN infected	72,276.61 ± 9,471.59 **a**	270.34 ± 61.55 **ab**	404.85 ± 65.62 **ab**	102.48 ± 15.41	5.64 ± 0.99 **b**
PION 93Y05	SCN resistant	SCN infected	64,221.68 ± 7,405.28 **a**	340.95 ± 53.61 **a**	535.50 ± 76.51 **a**	126.67 ± 14.19	7.19 ± 1.23 **b**

Table 8.19: Raw data of Soybean at R1 stage. Concentration is in pmol g^{-1}DWt. ND represents CKs that were not detected.

			FB				RB				NT				MES			
Genotype-Reps	**Treatment**	**SCN susceptibility**	**tZ**	**cisZ**	**DZ**	**iP**	**tZR**	**cisZR**	**DZR**	**iPR**	**tZNT**	**cisZNT**	**DZNT**	**iPNT**	**MeSZ**	**MeSiP**	**MeSZR**	**MeSiPA**
Lee74-1	Control	SCN susceptible	ND	75.56	ND	ND	ND	25.85	ND	44.35	ND	25.82	ND	43.41	359.89	881.56	14.56	8.62
Lee74-2	Control	SCN susceptible	ND	34.67	ND	3.98	3.20	1.74	ND	36.85	ND	4.00	ND	17.60	348.74	905.12	21.00	8.93
Lee74-3	Control	SCN susceptible	ND	28.91	ND	6.86	2.99	5.03	ND	38.99	ND	15.32	ND	86.38	567.67	1053.84	4.37	ND
Lee74-4	Control	SCN susceptible	ND	42.05	ND	4.24	ND	8.83	4.32	32.11	ND	5.99	ND	22.53	557.56	1326.39	21.86	17.24
Lee74-1	SCN infected	SCN susceptible	ND	216.76	ND	9.28	100.30	108.56	27.95	237.53	55.94	27.47	7.39	231.78	815.47	769.38	12.05	ND
Lee74-2	SCN infected	SCN susceptible	ND	215.27	ND	6.41	166.67	42.65	31.78	321.82	154.48	28.13	13.66	168.90	486.69	ND	9.50	8.51
Lee74-3	SCN infected	SCN susceptible	ND	180.16	ND	12.21	96.39	55.12	19.62	216.40	287.03	54.40	32.02	486.01	432.90	726.00	15.77	11.49
Lee74-4	SCN infected	SCN susceptible	ND	233.57	ND	16.86	140.66	32.27	21.58	161.51	404.70	44.32	37.40	412.08	481.06	ND	14.99	4.48
Essex-1	Control	SCN susceptible	1.55	194.12	5.94	0.52	0.30	19.28	3.81	4.05	ND	ND	ND	ND	67.21	38.27	3.85	0.38
Essex-2	Control	SCN susceptible	1.14	247.27	ND	0.40	8.19	33.01	9.90	13.28	ND	31.70	ND	ND	75.98	32.73	1.78	0.74
Essex-3	Control	SCN susceptible	3.34	110.10	ND	3.37	1.13	10.03	1.13	9.31	ND	ND	ND	ND	122.65	171.54	3.09	5.87
Essex-4	Control	SCN susceptible	ND	120.41	ND	0.78	0.00	18.19	4.14	4.38	ND	28.40	ND	ND	90.08	75.12	12.26	ND
Essex-1	SCN infected	SCN susceptible	3.25	736.85	3.05	2.55	197.38	93.12	35.85	224.00	288.85	53.89	11.92	833.61	203.65	143.08	42.31	1.61
Essex-2	SCN infected	SCN susceptible	15.89	1109.27	8.66	2.91	140.72	59.85	33.65	158.20	268.04	48.35	6.11	1568.58	106.44	175.66	8.57	1.51
Essex-3	SCN infected	SCN susceptible	8.93	344.08	5.52	46.13	193.59	99.54	35.63	240.84	921.01	141.31	40.58	3013.08	136.73	89.22	6.96	2.23
Essex-4	SCN infected	SCN susceptible	5.64	304.56	3.62	6.22	108.94	84.76	25.91	202.35	293.95	52.54	10.44	5522.40	111.72	65.95	24.92	2.66
PION P22T69R-1	Control	SCN resistant	ND	26.68	ND	5.95	10.19	1.80	14.39	22.95	ND	3.07	ND	4.65	281.24	1113.42	10.99	7.90
PION P22T69R-2	Control	SCN resistant	ND	9.74	ND	0.80	3.76	12.07	ND	26.80	ND	16.62	ND	3.29	134.52	389.62	9.66	3.25
PION P22T69R-3	Control	SCN resistant	ND	4.14	ND	5.47	ND	10.22	2.68	31.67	ND	5.81	ND	9.25	326.97	552.73	13.31	9.87
PION P22T69R-4	Control	SCN resistant	ND	26.35	ND	ND	6.21	14.38	14.76	20.99	ND	9.81	ND	17.47	529.42	917.90	5.27	3.59
PION P22T69R-1	SCN infected	SCN resistant	ND	27.19	ND	23.64	34.61	55.48	ND	167.82	24.86	26.80	ND	133.82	817.39	ND	21.66	6.94
PION P22T69R-2	SCN infected	SCN resistant	ND	28.15	ND	21.75	26.02	26.91	4.87	112.65	17.38	14.04	ND	160.57	550.11	ND	19.03	1.92
PION P22T69R-3	SCN infected	SCN resistant	ND	26.17	ND	9.18	7.13	12.84	ND	50.16	6.58	16.06	ND	208.67	523.69	1687.16	28.72	ND
PION P22T69R-4	SCN infected	SCN resistant	ND	197.84	ND	7.98	40.59	84.73	ND	173.25	2.30	5.59	ND	85.06	544.64	1125.34	10.24	7.84
Pion93Y05-1	Control	SCN resistant	ND	322.58	ND	13.80	3.60	25.09	1.32	13.58	ND	26.47	ND	0.00	44.06	61.25	1.39	ND
Pion93Y05-2	Control	SCN resistant	ND	204.56	6.43	3.86	ND	38.07	3.47	8.15	ND	20.55	ND	227.67	101.41	69.72	10.82	1.19
Pion93Y05-3	Control	SCN resistant	7.31	311.72	22.85	1.89	0.96	27.07	8.66	4.07	ND	14.12	ND	65.44	35.86	92.15	ND	ND
Pion93Y05-4	Control	SCN resistant	ND	79.13	12.07	1.13	0.60	21.61	0.74	3.42	ND	20.78	ND	62.05	61.41	65.66	5.43	2.25
Pion93Y05-1	SCN infected	SCN resistant	7.08	408.13	9.11	5.49	80.86	34.22	7.50	108.34	149.16	32.75	ND	840.97	116.59	120.93	3.27	ND
Pion93Y05-2	SCN infected	SCN resistant	14.00	448.86	4.89	3.63	63.64	58.48	7.41	129.13	92.93	19.34	ND	896.84	69.61	64.42	7.36	ND
Pion93Y05-3	SCN infected	SCN resistant	2.31	377.49	3.35	0.96	60.26	50.06	6.69	78.48	91.69	20.80	ND	1379.46	211.85	174.34	8.80	2.29
Pion93Y05-4	SCN infected	SCN resistant	3.14	412.44	3.03	3.68	29.97	79.23	5.23	72.47	59.60	25.56	ND	313.95	144.34	95.58	11.76	4.00

Table 8.20: Average of different FBCKs in soybean cultivars at R1 stage. Concentration is in pmol g^{-1}DWt. P value < 0.05. ND represents CKs that were not detected.

Cultivars	SCN susceptibility	Treatment	tZ	cZ	DZ	iP
Lee74	SCN susceptible	SCN non-infected	ND	45.3 ± 9.04 **c**	ND	3.77 ± 1.23 **NS**
Essex	SCN susceptible	SCN non-infected	1.51 ± 0.6 **b**	167.98 ± 28.04 **c**	1.49 ± 1.29 **b**	1.27 ± 0.61
PION P22T69R	SCN resistant	SCN non-infected	ND	16.73 ± 4.99 **c**	ND	3.05 ± 1.34
PION 93Y05	SCN resistant	SCN non-infected	1.83 ± 1.58 **b**	229.5 ± 49.15 **bc**	10.34 ± 4.2 **a**	5.17 ± 2.54
Lee74	SCN susceptible	SCN infected	ND	211.44 ± 9.72 **bc**	ND	11.19 ± 1.93
Essex	SCN susceptible	SCN infected	8.43 ± 2.38 **a**	623.69 ± 163.67 **a**	5.21 ± 1.09**ab**	14.45 ± 9.17
PION P22T69R	SCN resistant	SCN infected	ND	69.84 ± 36.95 **c**	ND	15.64 ± 3.55
PION 93Y05	SCN resistant	SCN infected	6.63 ± 2.31 **a**	411.73 ± 12.66 **ab**	5.1 ± 1.21 **ab**	3.44 ± 0.81

Table 8.21: Average of different RBCKs in soybean cultivars at R1 stage. Concentration is in pmol g^{-1}DWt. P value < 0.05.

Cultivars	SCN susceptibility	Treatment	tZR	cZR	DZR	iPR
Lee74	SCN susceptible	SCN non-infected	1.549 ± 0.78 **d**	10.36 ± 4.64 **e**	1.08 ± 0.94 **c**	38.08 ± 2.20 **c**
Essex	SCN susceptible	SCN non-infected	2.406 ± 1.68 **d**	20.13 ± 4.13 **de**	4.74 ± 1.60 **c**	7.75 ± 1.91 **c**
PION P22T69R	SCN resistant	SCN non-infected	5.040 ± 1.85 **d**	9.61 ± 2.37 **e**	7.96 ± 3.34 **c**	25.60 ± 2.04 **c**
PION 93Y05	SCN resistant	SCN non-infected	1.291 ± 0.69 **d**	27.96 ± 3.08 **cde**	3.54 ± 1.56 **c**	7.31 ± 2.02 **c**
Lee74	SCN susceptible	SCN infected	126.004 ± 14.59**b**	59.65 ± 14.69 **ab**	25.23 ± 2.44 **b**	234.32 ± 28.82 **a**
Essex	SCN susceptible	SCN infected	160.156 ± 18.55**a**	84.32 ± 7.53 **a**	32.76 ± 2.02 **a**	206.35 ± 15.48 **a**
PION P22T69R	SCN resistant	SCN infected	27.087 ± 6.32 **d**	44.99 ± 13.81**bcd**	1.22 ± 1.06 **c**	125.97 ± 24.89 **b**
PION 93Y05	SCN resistant	SCN infected	58.682 ± 9.16 **c**	55.50 ± 8.12 **abc**	6.71 ± 0.45 **c**	97.11 ± 11.47 **b**

Table 8.22: Average of different NTCKs in tested soybean cultivars at R1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05. ND represents CKs that were not detected.

Cultivars	SCN susceptibility	Treatment	tZNT	cZNT	DZNT	iPNT
Lee74	SCN susceptible	SCN non-infected	ND	12.78 ± 4.33 b	ND	42.48 ± 13.57b
Essex	SCN susceptible	SCN non-infected	ND	15.02 ± 7.54 b	ND	ND
PION P22T69R	SCN resistant	SCN non-infected	ND	8.83 ± 2.55 b	ND	8.67 ± 2.77 b
PION 93Y05	SCN resistant	SCN non-infected	ND	20.48 ± 2.19 b	ND	88.79 ± 42.16b
Lee74	SCN susceptible	SCN infected	225.538 ± 66.00b	38.58 ± 5.68 b	22.62 ± 6.22 a	324.69 ± 64.50b
Essex	SCN susceptible	SCN infected	442.963 ± 138.09a	74.02 ± 19.45 a	17.26 ± 6.81 a	2,734.42 ± 895.22a
PION P22T69R	SCN resistant	SCN infected	12.779 ± 4.44 c	15.62 ± 3.78 b	ND	147.03 ± 22.36b
PION 93Y05	SCN resistant	SCN infected	98.347 ± 16.12bc	24.61 ± 2.62 b	ND	857.80 ± 188.70b

Table 8.23: Average of different 2MeSCKs in soybean cultivars at R1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05.

Name	SCN susceptibility	Treatment	2MeSZ	2MeSiP	2MeSZR	2MeSiPR
Lee74	SCN susceptible	SCN non-infected	458.47 ± 52.14 ab	1,041.73 ± 88.56 a	15.45 ± 3.49abc	8.70 ± 3.05 a
Essex	SCN susceptible	SCN non-infected	88.98 ± 10.54 c	79.41 ± 27.81 c	5.24 ± 2.06 c	1.74 ± 1.20 b
PION P22T69R	SCN resistant	SCN non-infected	318.04 ± 70.62 b	743.42 ± 143.37ab	9.81 ± 1.46abc	6.15 ± 1.41 ab
PION 93Y05	SCN resistant	SCN non-infected	60.69 ± 12.63 c	72.19 ± 5.95 c	4.41 ± 2.10 c	0.86 ± 0.47 b
Lee74	SCN susceptible	SCN infected	554.03 ± 76.192 a	373.846 ± 187.080bc	13.078 ± 1.245abc	6.120 ± 2.161 ab
Essex	SCN susceptible	SCN infected	139.63 ± 19.345c	118.476 ± 21.637 c	20.691 ± 7.161 a	2.000 ± 0.235 b
PION P22T69R	SCN resistant	SCN infected	608.96 ± 60.371 a	703.123 ± 365.321ab	19.913 ± 3.306 ab	4.174 ± 1.650 ab
PION 93Y05	SCN resistant	SCN infected	135.60 ± 25.746c	113.819 ± 20.135 c	7.797 ± 1.529 bc	1.575 ± 0.843 b

Table 8.24: Average of FBs, RBs, NTs, 2MeSCKs and glucosides in soybean cultivars at R1 stage. Concentration is in pmol g^{-1}DWt. *P* value < 0.05. ND represents CKs that were not detected.

Name	SCN susceptibility	Treatment	FB	RB	NT	2MeS	Glucosides
Lee74	SCN susceptible	SCN non-infected	49.07 ± 7.89 **c**	51.07 ± 5.60 **c**	55.26 ± 16.20 **b**	1,524.33 ± 135.69 **a**	ND
Essex	SCN susceptible	SCN non-infected	172.24 ± 27.88 **c**	35.03 ± 8.54 **c**	15.02 ± 7.54 **b**	175.38 ± 39.34 **b**	ND
PION P22T69R	SCN resistant	SCN non-infected	19.78 ± 4.98 **c**	48.21 ± 2.64 **c**	17.49 ± 3.56 **b**	1,077.42 ± 190.22 **a**	2.57 ± 1.64 **b**
PION 93Y05	SCN resistant	SCN non-infected	246.83 ± 51.42 **bc**	40.10 ± 4.28 **c**	109.27 ± 41.64 **b**	138.15 ± 13.98 **b**	ND
Lee74	SCN susceptible	SCN infected	222.63 ± 10.31 **bc**	445.20 ± 40.30 **a**	611.43 ± 134.16 **b**	947.07 ± 233.79 **a**	277.21 ± 46.12 **a**
Essex	SCN susceptible	SCN infected	651.79 ± 161.07 **a**	483.58 ± 38.70 **a**	3,268.67 ± 927.34 **a**	280.80 ± 35.35 **b**	ND
PION P22T69R	SCN resistant	SCN infected	85.47 ± 34.87 **c**	199.26 ± 43.88 **b**	175.43 ± 25.37 **b**	1,336.17 ± 332.19 **a**	20.60 ± 10.63 **b**
PION 93Y05	SCN resistant	SCN infected	426.90 ± 15.49 **b**	217.99 ± 14.35 **b**	980.76 ± 194.01 **b**	258.79 ± 45.62 **b**	ND