# ELUCIDATION OF PHYSIO-CHEMICAL MECHANISM ASSOCIATED WITH BANANA PAPER IN THE MANAGEMENT OF POTATO CYST NEMATODES

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**FEBRUARY, 2021**

## DECLARATION

I hereby declare that this is my original work and has not been presented for the award of degree in any other university.

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## DEDICATION

It is with deep gratitude that I dedicate this work to my parents; Mr. Dalmas Ochola Owino and Mrs. Millicent Akinyi Ochola, and sisters Maureen, Marylyn and Brenda.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ANOVA | Analysis of variance |
| EPPO | European and Mediterranean Plant Protection Organization |
| FAO | Food and Agriculture Organization |
| HF | Hatching factors |
| HI | Hatching inhibitors |
| *icipe* | International Centre of Insect Physiology and Ecology |
| IPM | Integrated pest management |
| J2 | Second stage infective juvenile |
| PCN | Potato cyst nematodes |
| PPNs | Plant parasitic nematodes |
| PRE | Potato root exudates |
| RKN | Root knot nematodes |
| SGA | Steroidal glycoalkaloids |
| UPLC-QqQ-MS/MS | Ultra-performance liquid chromatography coupled to a triple quadrupole tandem mass spectrometry |
| VOC | Volatile organic compounds |
| ‘W&P’ | ‘Wrap and Plant’ |

## ABSTRACT

Potato production in Kenya is threatened by the occurrence of the widespread potato cyst nematode (PCN), which causes yield losses of more than 80%. Recent attempts to assess novel management options for PCN in the country demonstrated the effectiveness of the "Wrap & Plant" (W&P) technology to increase yields under heavy PCN infestations. This innovation consists of a field deployable biodegradable matrix produced from banana fibre (banana paper) that is impregnated with abamectin (nematicide). Farms where potato seeds were wrapped with the abamectin-treated and the untreated banana paper (paper control) increased yields by 4-fold and 3-fold, respectively, and showed decline of 83% and 43% of the PCN inoculum in the soil. The current study sought to elucidate the underlying mechanism associated with the banana paper in management of PCN. We hypothesised that banana paper acts as a physical and/or chemical barrier, hampering plant-nematode communication, which is important for PCN hatching and host infection. Behavioural experiments indicated that the untreated and treated banana paper reduced PCN hatching by 60% and 80%, respectively. The banana paper also inhibited chemotactic response of the PCN juveniles, reducing their attraction by 99 and 94% in treated and untreated banana paper, respectively. An evaluation of the effect of the banana paper on PCN development using pot trials also showed a reduction in formation of females when the potatoes were wrapped with the banana paper compared to when potatoes were not wrapped, suggesting a lowered reproduction rate in wrapped plants. Chemical analysis by ultra-performance liquid chromatography, coupled to triple quadrupole mass spectrometry (UPLC-QqQ-MS) of the potato root exudates (PRE) detected the tetranortriterpene solanoeclepin A (**2**), steroidal glycoalkaloids α-solanine (**3**) and α-chaconine (**4**); the steroidal alkaloids solanidine (**24**), solasodine (**27**), and tomatidine (**28**); phytohormones, zeatin (**10**) and methyl dihydrojasmonate (**23**); and amino acids, tryptophan (**22**), tyrosine (**20**) and phenylalanine (**21**). Of these compounds, the steroidal glycoalkaloids and steroidal alkaloids showed hatch stimulating activity when tested on PCN *G. rostochiensis*. Potato root exudate exposure to banana paper, revealed that the paper matrix adsorbs most of the PRE components inclusive of the identified PCN hatching factors. Further experiments to understand the interaction between the banana paper and PRE showed a similar interaction between pure cellulose powder and PRE, suggesting that adsorption of the polar components of the PRE by hydrogen bonding on the cellulose matrix of the banana paper could be the mechanism underlying the observed effect. This research provides new insights on how PCN could be managed at the rhizosphere level by disrupting the chemical signalling with its hosts plant using a lignocellulosic matrix that can adsorb the hatching semiochemicals.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background information

Plant parasitic nematodes (PPNs) are obligate worms that rely on living plants for reproduction and development (Oka *et al.*, 2000). They pose an a major constraint to food security and cause economic losses estimated at 80 billion USD per year (Nicol *et al.*, 2011). About 4100 species of PPNs have been identified, with root knot nematodes (RKN) (*Meloidogyne* species) and cyst nematodes (*Globodera* and *Heterodera* species) ranking first and second most important genera with regard to scientific and economic importance (Jones *et al.*, 2013).

Cyst nematodes are sedentary endoparasites that spend most of their life inside the host. Their attack diverts the host plant nutrients to the nematode as well as result in physical damage to plant tissue, as the nematodes migrate through the roots, which can also cause potential secondary infections (Jones *et al.*, 2013). This leads to reduced water and nutrient uptake, resulting in decreased crop yields. To ensure effective penetration of root tissue, the nematodes secrete effectors, comprised of proteins, which are capable of altering the host cell structure (Mejias *et al.*, 2019). Additionally, they also suppress host plant defenses (Haegeman *et al.*, 2012; Mejias *et al.*, 2019; Yang *et al.*, 2019), increasing the susceptibility of the plant to attack by other pests and pathogens.

Most cyst nematodes have very limited host range and rely on chemical cues originating from these hosts as stimulants for hatching and host location (Devine and Jones, 2003;

Perry, 2005; Rawsthorne and Brodie, 1986). They are highly specialized parasites that form cysts (a durable protective casing) around their eggs, which enable them to endure harsh climatic and environmental conditions for prolonged periods until compatible hosts are available (Christoforou *et al.*, 2014; Maurice *et al.*, 2018). This makes their control a major challenge, which underlie their economic importance (Jones *et al.*, 2013; Maurice *et al.*, 2018). The most damaging species of cyst nematodes include potato cyst nematode (*Globodera rostochiensis* and *G. pallida*), soybean cyst nematode (*Heterodera glycines*), and cereal cyst nematode (*Heterodera filipjevi* and *H. avenae*) (Turner and Rowe, 2006).

Potato cyst nematodes (PCN) are of great economic importance since their introduction in Europe in the mid 1880's. They are believed to have originated from the Andean region in South America (Grenier *et al.*, 2010), and subsequently spread to all major potato growing regions in the world (Hockland *et al.*, 2012). There are two common PCN species i.e. the golden cyst nematode *G. rostochiensis,* and the pale potato cyst nematode *G. pallida* (Sullivan *et al.*, 2007). These nematodes differ genetically, as well as in host interactions and the environmental optima conducive for different phase in the life cycle (Hodda and Cook, 2009). They attack the roots of some *Solaneceae* plants that include; potato (*Solanum tuberosum*) and to a lesser extent, tomato (*S. lycopersicum*), and eggplant (*S. melongena*) (Ryan *et al.*, 2000). Potato cyst nematodes have a severe yield reduction potential that has led to their classification as quarantine pests in many countries. In UK alone, an estimated 9% loss of potato product is as a result of PCN damage (Turner and Rowe, 2006), and being a quarantine pest, it is also a significant limitation on international trade (Eberlein *et al.*, 2016).

Potato (*S. tuberosum*) is a widely consumed vegetable crop, globally. In Kenya, it is the second most important food crop after maize (Abong and Kabira, 2013; Janssens *et al.*, 2013) with a nationwide annual production of 9–10 t/ha valued at USD 500 million (CIP, 2019), and mainly grown by small-holder farmers. In the recent years, potato production in the region has declined by more than 60% despite increase on the arable land under cultivation (area harvested) (Figure 1.1) (FAOSTAT, 2017). This is due to a number of factors, among which is the occurrence of PCN that was first reported in the country in 2015 (Mwangi *et al.*, 2015). So far, the two species of PCN i.e., *G. rostochiensis* and *G. pallida* have been reported with the latter being more restricted in distribution (Mburu *et al.*, 2018).

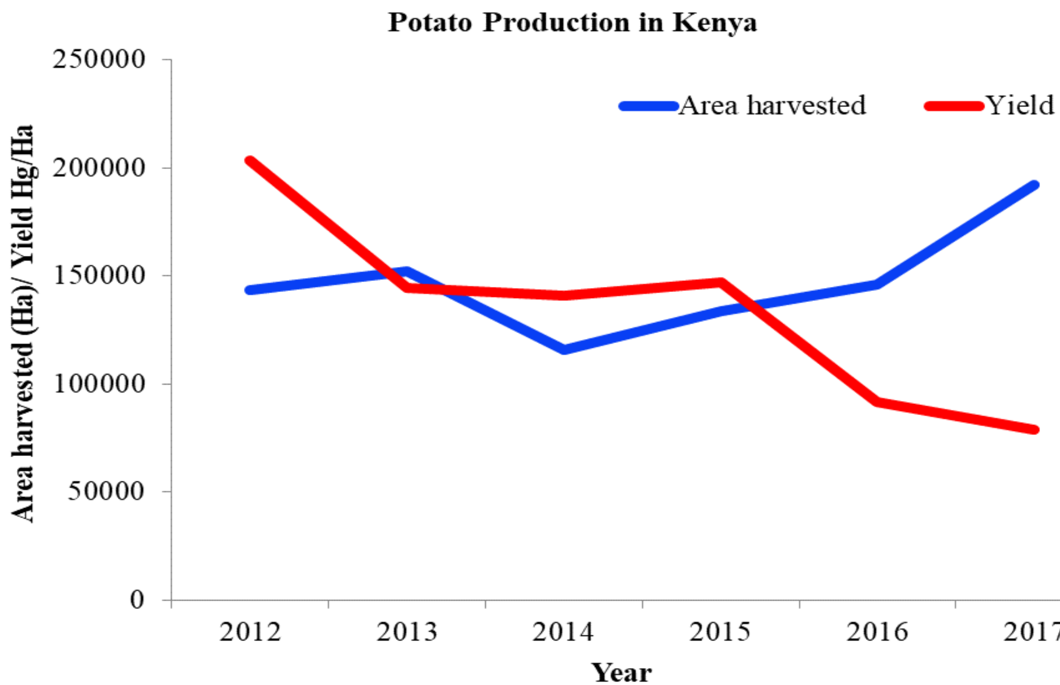



**Figure 1.1:** Statistics on the decline of potato production in Kenya from year 2012-2016 (FAOSTAT, 2017).

Current PCN management strategies involve use of botanicals, synthetic nematicides, biological control agents, genetic resistance, crop rotation, trap crops, fallow rotation, organic soil amendments and/or intercropping (Ochola *et al.,* 2020a, 2020b). The effectiveness of these strategies varies from one to another, but they all suffer some limitations. Several synthetic nematicides have been banned due to their adverse environmental and toxicity effects (Khalil, 2013). Despite resistant cultivars having been identified as the most effective method for PCN management, the species-specific resistance response of some of the resistance genes (*R*-genes) to certain nematode species/pathotypes allows other species, when present, to reproduce (Ochola *et al.,* 2020a, 2020b). The ability of the cysts to endure as diapausing structures in the soil in the absence of a host with low percentage of spontaneous hatch (Sparkes, 2016), also limits the effectiveness of crop rotation, even in the absence of a host. The socio-economic and food security context in Kenya also makes it very challenging for smallholder subsistence farmers to adopt some of these strategies. Hence, it is crucial to develop other cheaper, environmentally benign and yield increasing control measures.

A recent exploratory research investigated the 'Wrap and Plant' ('W&P') technology, as an alternative mechanism to manage plant parasitic nematodes (PPNs) (Cao *et al.*, 2016; Cortada *et al.*, 2018; Pirzada *et al.,* 2020). This is an innovation that uses a banana fiber matrix, 'banana paper' impregnated with a nematicide (abamectin) developed to control PPNs (Cao *et al.*, 2016; Cortada *et al.*, 2018; Pirzada *et al.,* 2020). This novel approach is able to reduce the required active ingredient for crop protection by 1000 times compared granular application. Additionally, it also offers a more targeted delivery thus exposing

the target organism to higher concentrations of the active ingredient and minimizing the detrimental effects on non-target organisms and the environment. Field trials to evaluate this technology against PCN in Kenya demonstrated that when the abamectin impregnated paper (treated banana paper) was used to wrap the potato seeds before planting, there was a 4-fold increase in potato yields (grams per plant) compared to the control which represented the conventional farmers practice (Cortada *et al.*, 2018). This increment was 35.8% more than what was observed when abamectin alone was applied in the fields. In parallel to the increase in yields, the cyst populations in the soil also decreased by 85%. Surprisingly, when the banana paper was used alone (untreated banana paper), the yields increased by 3-folds and the population of cysts in the soil also decreased by 43% (Cortada *et al.*, 2018). These results suggested other pathways associated with the banana fiber paper that facilitated incremental control of PCN. The current study sought out to elucidate from a chemical ecology perspective, the physical and/or chemical mechanisms associated with the banana fiber paper in the management of PCN.

## 1.2 Statement of the problem

Potato cyst nematodes (PCN) cause huge economic losses to potato production worldwide. In the recent years, potato production in Kenya has been declining regardless of the increasing cultivation areas. This could be attributed to several factors, among them PCN, which was recently reported in the country. Most of the available control strategies are not readily available or easily adoptable to the small-holder farmers in Kenya who make up 98% of potato farmers. As such, there is need to develop more

efficient control methods that can suit small scale farming systems. The relative efficacy of banana fiber paper in reducing PCN populations in potato plants and increasing potato yields was previously demonstrated, under field trials during two consecutive cropping seasons in Nyandarua County. The method is environmentally benign and affordable to small holder farmers. However, there is limited information on its mode of action and the underlying principles responsible for this management approach. This study sought to elucidate the chemical ecology mechanism associated with banana paper in the management of PCN.

### 1.3 Justification of the study

Most of the current measures used to control PCN have major limitations. For example, use of nematicides is expensive for small-holder farmers and the indiscriminate use of hazardous chemicals can lead to both human and environmental toxicity. Effective crop rotation requires growing non-host crops in infested fields for long periods of time due to the prolonged existence of PCN cysts in the soil while most small holder farmers tend to grow potatoes all year round. The use of potato resistant varieties, even though considered the most effective management method, is not effective in mixed populations since the resistance genes (*R*-genes) are species/pathotype-specific. Additionally, PCN-resistant potato varieties in Kenya are not readily available, and in the few areas where these resistant varieties are available, they are not used due to farmers' preference and market demands of traditionally used varieties. In Kenya, farmers also tend to cultivate potatoes all year round with limited rotations that have a very limited impact on PCN management. These limitations call for more sustainable and effective management

strategies. The ‘W&P’ technology has been demonstrated to be effective in increasing potato production in PCN infested soils in addition to reducing cyst population in the soil. However, the mechanism associated with this effect has not been studied. PCN is only able to hatch and locate the host plant with the help of hatching factors (HFs) and chemo-attractants present in the host root exudates. A better understanding on the role of the banana paper in the PCN – potato plant interaction would facilitate enhancement of its effects to improve its performance and broaden its application to other PPNs.

**1.4 Hypotheses**

i. The banana paper interacts with the PCN hatching factors (HFs) present in the potato root exudates (PRE) delaying hatching of the eggs in the cyst.

ii. The banana paper acts as a physical barrier slowing down diffusion of PRE in the soil and preventing the chemotactic migration of the second stage juvenile nematodes (J2) in the soil.

iii. Wrapping potato seeds with banana paper reduces the number of PCN attacking the potato plant and the rate at which cysts develop compared to an unwrapped potato.

iv. Exposing banana paper to PRE results in structural modifications of the HFs or their effective adsorption on the paper.

## 1.5 Objectives

### 1.5.1 General objective

To elucidate the mechanism(s) associated with banana fiber paper in the management of potato cyst nematode (PCN).

### 1.5.2 Specific objectives

i. To evaluate the hatching response of PCN eggs to PRE in the presence of banana paper.
ii. To evaluate the chemotaxis response of hatched second stage juvenile nematodes (J2) to PRE in the presence of banana paper.
iii. To determine the effect of banana fiber paper on the development of PCN.
iv. To determine the chemical composition of PRE before and after exposure to banana fiber paper.

## 1.6 Significance of study

The study sought to elucidate the mechanism associated with the 'W&P' technology in the management of PCN to provide a clearer understanding of its mode(s) of action that would enable improving its effectiveness and widening its application to other PPNs.

## 1.7 Scope and limitations

Though some studies have shown involvement of volatile cues in host location of PCN, this study focused on the possible roles of non-volatile cues and their characterization. The study also evaluated the chemical interaction between potato and only one species of PCN, i.e. *G rostochiensis*, which is the most prevalent in Kenya (Mburu *et al*., 2020) The

effect of banana paper on PCN-potato plant interaction was focused only on possible chemical interaction(s). The study only focused on potato variety ‘Shangi’ which is susceptible to PCN *G. rostochiensis* and is the most widely grown in PCN infested areas in Kenya.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Production and importance of potatoes

Potato (*Solanum tuberosum*) is the fourth most important food crop in the world, behind rice, wheat and maize, with an annual production of approximately 388 million tons in 2016 (FAOSTAT, 2017; Potatopro, 2019). Potatoes are thought to have originated from South America, and subsequently spread to other parts of the world where it's a major staple food (Evans and Stone, 1977). It grows under tropical, sub-tropical and temperate conditions with optimum yields at mean daily temperatures between 18 and 20°C (Luc *et al.*, 2005). A potato growing season can take up to 90 days in the tropical and 150 days in temperate regions depending on the potato variety (Bradshaw and Ramsay, 2009).

Globally, China is the leading potato producing country with an annual production of approximately 99 million tonnes (FAOSTAT, 2017). Its followed by India, Russian Federation, Ukraine, United states, Germany and Bangladesh who together produce more than half of the global potatoes ( FAOSTAT, 2017; Potatopro, 2019).

In Africa, potatoes were introduced in the turn of the 20$^{th}$ century (FAO, 2008). Currently, Africa produces approximately 24 million tons of potatoes annually with Algeria and Egypt being the biggest producer, with approximately 4.6 and 4.3 million tonnes in 2017 for exports, local consumption and processing ( FAOSTAT, 2017). Worldwide, Egypt also ranks among the major exporter of fresh and frozen potato products (Defauw *et al.*, 2012). South Africa is also a major exporter of potatoes. In

South Africa, potatoes are valued at € 42 million with an annual production of 2 million metric tons in 50,000 ha (Onkendi and Moleleki, 2013).

Potatoes were introduced in Kenya, in the mid 1800's (FAO, 2008) and is currently the second most important food crop after maize (Janssens *et al.*, 2013). Its production is mainly in the highlands of Eastern, Rift valley and Central regions, slopes of Mt. Kenya and the Mau range among other regions. Potatoes in Kenya are mainly grown by small holder farmers and consumed locally (Muthoni *et al.*, 2013; Onkendi and Moleleki, 2013). It plays a major role in national food security and poverty alleviation through the creation of employment and income generation. In Kenya between 500,000 and 780,000 people are directly involved in potato farming activities (Janssens *et al.*, 2013) and 2.5 million people across the value chain rely on it as a source of income (Abong and Kabira, 2013).

Currently potato production in Kenya is at an average of 9-10 t/ha, which is below its potential of 20-40 t/ha (Janssens *et al.*, 2013). Recently, potato production in Kenya has been on decline (Fig.1.1). This could be attributed to a number of factors, among them potato cyst nematodes (PCN) that was first reported in the country in 2015 (Mwangi *et al.*, 2015).

### 2.2 Potato cyst nematodes (PCN)

Potato cyst nematodes are a key constraint to potato production worldwide. It can be responsible for 80% to total crop failure under high infestations. PCN is thought to have

originated from South America in the late 1800s (Dossey, 2011; Holden, 2015) and co-evolved with potatoes. PCN also infects other solanaceous plants like eggplant (*Solanum melongena*), tomatoes (*Solanum lycopersicum*), Capsicum spp., or woody nightshade but to a lesser extent (Nicol *et al.*, 2011; Torto *et al.*, 2018a).

There are 2 major species of PCN, i.e., *Globodera rostochiensis* (Figure 2.1 A) and *Globodera. pallida* (Figure 2.1 B). The two species can be distinguished using morphology of the infective stage juvenile and the color of female adults, among others. *G. pallida* has a longer body length and a stylet compared to *G. rostochiensis*. Adult females of the latter move through three colors i.e. white, yellow and brown while *G pallida* do not undergo the yellow phase (EPPO, 2013; Sasaki-Crawley, 2013). Apart from the morphological differences, there are also several molecular techniques that are used in their identification.

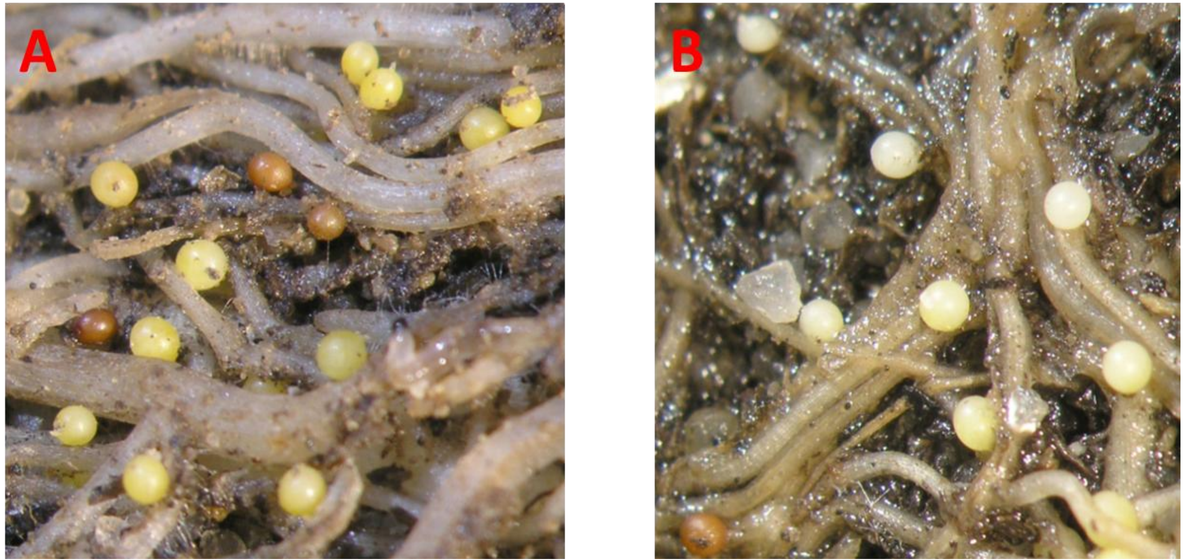



**Figure 2.1:** Potato cyst nematode species *Globodera rostochiensis* (A) and *Globodera. pallida* (B) as viewed under a microscope. Photos by X. Wang, (Grenier and Mimee, 2017)

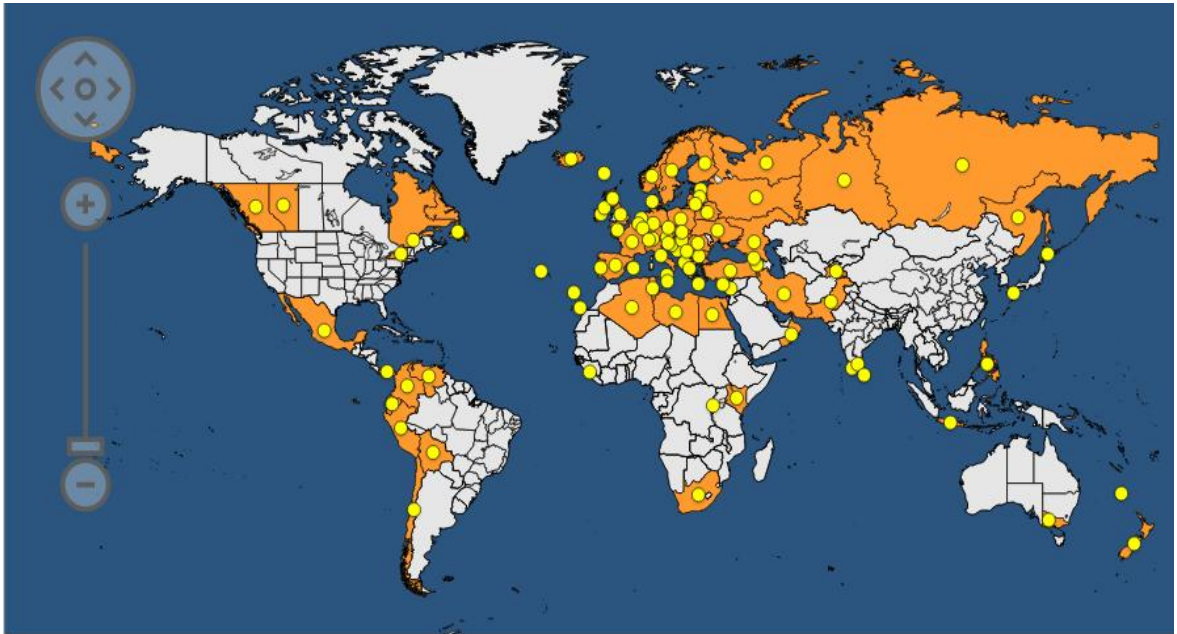


**Figure 2.2:** Worldwide distribution of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*). Yellow dots represent countries where PCN has been reported (EPPO,2019)

PCN is a quarantine pest in over 100 countries (Figure 2.2) and this seriously affects production and trade of potatoes (Eberlein, 2016; EPPO, 2018). Evaluation of economic losses caused by PCN is difficult because the damage is dependent on environmental, biological and cultural factors. In some parts of the world like UK, where PCN is well studied, losses impacted on potato yields has been estimated at 9% of potato products, corresponding to a market value of USD 70 million annually (Coyne *et al.*, 2018; Nicol *et al.*, 2011). Other studies in the European Union have also shown PCN to be responsible for losses worth approximately € 300 million annually (Haydock and Evans, 1998; Sasaki-Crawley, 2013). Yield losses of PCN are usually proportional to the initial nematode populations (Greco *et al.*, 1982; Seinhorst, 1982), with an initial population of 20 viable egg/g of soil estimated to cause up to 2 t/ha of potato loss (Brown and Sykes, 1983).

In Africa, *G. rostochiensis* has been reported in a few countries, including South Africa, Algeria, Tunisia, Libya, Morocco, Egypt, Sierra Leone, Zimbabwe (EPPO, 2013) and more recently Kenya (Mwangi *et al.*, 2015), Rwanda (Niragire *et al.*, 2019) and Uganda (Mburu *et al.*, 2020) (Figure 2.2). *G. pallida* now dominating major potato growing areas the UK has only been reported in a few countries in Africa (CABI, 2020) including Kenya (Mburu *et al.*, 2018). However, data on the losses caused by this devastating nematode in Africa are not available.

### 2.2.1 Potato cyst nematodes in Kenya

PCN was first reported in Kenya in 2015 during a national survey targeting potato crops. The PCN species found in these areas was identified as *G. rostochiensis* (Mwangi *et al.*, 2015). Following this report, a nationwide survey was conducted in 20 major potato growing regions in the country. This survey reported PCN prevalence at an average of 82% and also made the first report of *G. pallida* in the country (Mburu *et al.*, 2018).

Figure 2.3 shows the extent of PCN infestation in farms across the country, with more color intensity indicating higher level of infestation. The prevalence of PCN in Kenya is high and this could be attributed to several factors among which is the lack of clean planting materials in the country. Kenya produces less than 1% of the nation's demand for certified seeds, leading to a shortage of clean seeds that forces farmers to obtain seeds from other informal sources like local markets, neighbors and supplies from individual farms (Muthoni *et al.*, 2013). These informal systems are what enhance the spread of soil borne pathogens and pests. Repeated cultivation of potatoes on a farm with limited rotation and sharing of farm equipment without proper sanitation is also common practices in Kenya that can enhance PCN spread.

The current potato production in Kenya is only at 24.75% of its annual potato production potential. Yield loss due to PCN considering continuous cultivation of PCN susceptible cultivar like Shangi has been estimated at 5.1-21.8 t/ha per cropping season (Mburu et. al., 2020). Other potato varieties grown in Kenya include; Manitou, Markies, Destiny, Desirée and Jelly (Mburu et. al., 2020)

**Figure 2.3:** Map of Kenya showing potato cyst nematode (PCN) prevalence and distribution- from Mburu *et al.* (2020)

### 2.3.2 Life cycle of potato cyst nematodes

On average, a PCN cyst contains about 500 eggs (Lambert and Bekal, 2002) that hatch under favorable environmental conditions and on stimulation by chemical signals exuded by the roots of the host plant to produce second stage juvenile nematodes (J2) (Figure 2.4

i ) (Būda and Čepulytė-Rakauskienė, 2011). To complete its life cycle, PCN must undergo four juvenile stages before developing into an adult (Figure 2.4).

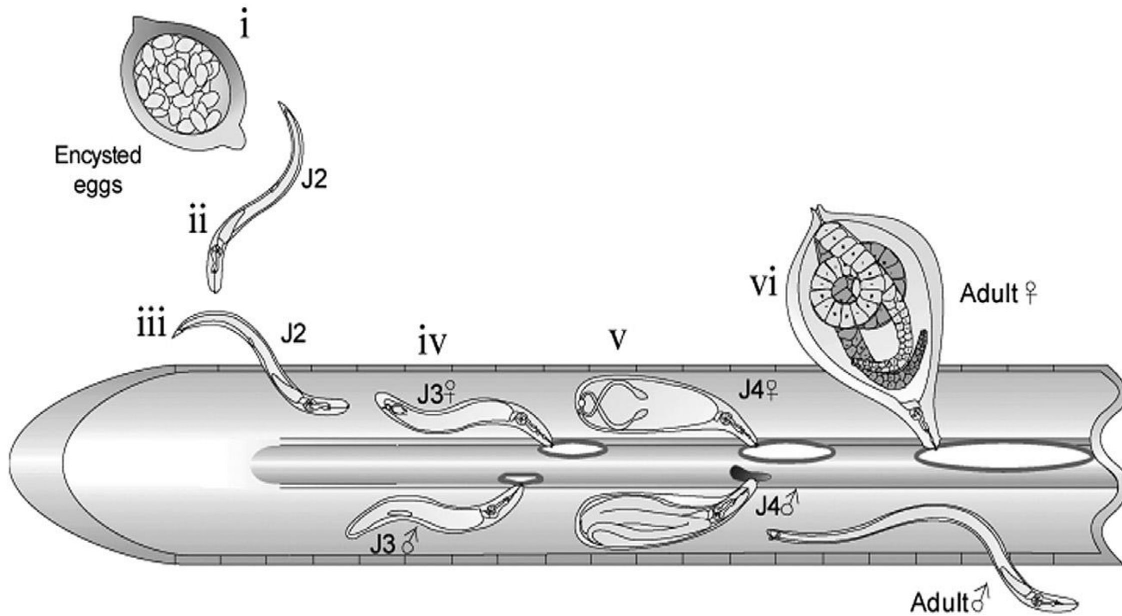



**Figure 2.4:** Life cycle of *Globodera* species (Bohlmann, 2015).

The hatched J2 locates the host plant by exploiting the gradients of chemical released by the host plant root system (Figure 2.4 ii ) (Perry, 2005). It then invades the host plant through the soft tissues present in the root tip and migrates intra-cellularly towards the vascular cylinder using its stylet to cut through the cell walls. Once in the pericycle, the J2 finds suitable cell where it establishes a feeding site (syncytium) by injection of esophageal enzyme secretions, which trigger a series of metabolic transformations leading to the cells' hypertrophy and to a partial degradation of the cell walls. This triggers a dozen or more neighboring cells to undergo a similar process, which merge and form a multinucleate protoplast or syncytium (Plantard *et al.*, 2008; Sobczak and Golinowski, 2011).

Once the syncytium is established, the J2 molts into a J3 then J4 (Figure 2.4 iv and v) and finally into either a male or female adult (Figure 2.4 vi ) (Jones *et al.*, 2013). The female nematode remains sedentary and saccate while feeding and extracting nutrients from the plant (Sharma and Sharma, 1998). Its body swells, increasing its reproductive capacity and allowing it to break through the root surface while still remaining attached to the roots (Sharma and Sharma, 1998).

The adult male becomes vermiform and actively moves out of the root where it locates a female with the aid of a pheromone produced by the female. It fertilizes the females who start to develop eggs in them. On egg maturation, the female nematodes die and their skins hardens to form protective covers (cysts) around the eggs (Christoforou *et al.*, 2014). During harvesting the cyst detaches from the roots remaining in the soil where it can survive for over 20 years in the absence of a host plant (Brodie and Mai, 1989; Duceppe *et al.*, 2017). PCN life cycle can take up to 3 months depending on the potato variety, or the temperatures in the soil, among others.

### 2.2.3 Potato cyst nematodes hatching

Potato cyst nematode is a specialized plant parasite (Oka *et al.*, 2000) whose life is synchronized with that of its host to optimize the chances of a successful invasion. This synchrony is possible due to its ability to form cysts enabling it to remain dormant in the soil for prolonged periods of time until a suitable host is present. Hatching of PCN juveniles can be spontaneous (Devine and Jones, 2001) to a lesser effect; although, substantial hatch is reliant on chemical signals (HFs) exuded by roots of host plants

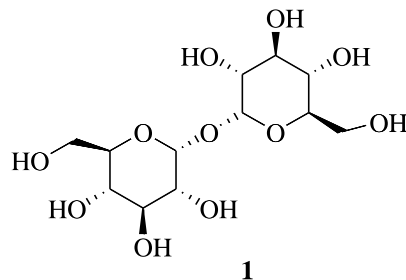
(Duceppe *et al.*, 2017). A potato plant 80 cm away, has been shown to activate juveniles resulting in hatching. This illustrates the high mobility of PRE in the soil (Turner and Rowe, 2006). Tsutsumi (1976) also demonstrated the stability of these HFs in the soil where their activity was still observed 100 days after removing the plant.

#### 2.2.3.1 Potato cyst nematode hatching mechanism

Potato cyst nematode display a double protection system including: a protective encasing of the cyst itself (body of a dead female), and the eggshell that protects the unhatched juvenile (Fernandez *et al.*, 2015). The cyst wall is a robust but permeable structure that acts as an interface between the nematode and its external environment. Eggs enclosed in a cyst can survive extreme temperatures and prolonged desiccation (Torto *et al.,* 2018a). The cyst wall of *G. rostochiensis* is comprised of mainly proteins, lipids, carbohydrates, glucosamine, polyphenols and ash (Clarke, 1968).

Hatching of PCN is almost completely reliant on host root diffusates. The first stage juvenile (J1) develops from an embryo and molts into the J2 within the egg (Moens *et al.*, 2018). Hatching of the J2 involves physico-chemical alteration of the eggshell structure that is comprised of three layers: the outer lipoprotein layer, middle chitinous layer and an inner semi-permeable lipid layer (Burgwyn *et al.*, 2003). Although permeable to respiratory gases, the lipid layer blocks the passing of water-soluble molecules until its permeability is altered (Perry and Clarke, 1981). As such, unhatched eggs of *G. rostochiensis* get into a state of partial hydration, with a water content of 67% due to the osmotic pressure generated by trehalose (**1**), found in the perivitelline fluid surrounding

the J2. Trehalose is a disaccharide associated with *G. rostochiensis* survival and hatching (Perry and Wharton, 2011) and its accumulation within the egg creates osmotic stress that protects the J2 against environmental shocks by inducing quiescence and inhibiting motility (Duceppe *et al.*, 2017). On the other hand, cyst nematode species, such as *H. schachtii,* readily hatch in water without the need for specific cues. Eggs of these species are known to have lower osmotic pressures, due to a lower concentration of trehalose, compared with species such as *G. rostochiensis* or *G. pallida* (Perry *et al.*, 1980). Reduction in the concentration of trehalose (**1**) is a pre-requisite for hatching, which occurs following a change in the permeability of the inner lipid layer of the eggshell. Alteration of the eggshell's permeability is a calcium-mediated process that involves HF binding or displacement of internal $Ca^{2+}$ (Atkinson *et al.*, 1980).




When diapause terminates, the J2 becomes rehydrated, and then a series of metabolic changes are initiated in the egg (Atkinson and Ballantyne, 1977a, 1977b). About three days after exposure of the cyst to root diffusates of a suitable host, vigorous J2 movement can be observed in the egg (Doncaster and Shepherd, 1967). The hydrated J2 then leaves the egg through a slit in the eggshell, made by using movements of the head and thrusting of the stylet. It then emerges from the cyst through the vulvar fenestra or anus to reach the soil. Hatching of *H. glycines* has been shown to be mediated by a divalent cation

($Zn^{2+}$) dependent enzyme, leucine aminopeptidase, found in the egg supernatant, activity of which is not enhanced by the presence of root diffusates (Tefft and Bone, 1985a;1985b). Chitinase gene has been reported to be expressed in hatched *G. pallida* J2 but not in the dormant J2, indicating that it is involved in the degradation of the middle chitin layer of the eggshell during hatching (Cotton *et al.*, 2014).

PCN are protected by a double system, i.e. the cyst, which is the protective cover of the eggs and the egg which protects the juveniles until they hatch (Almeida-Engler *et al.*, 2015). Dry cysts do not contain free water enabling them to survive freezing temperatures (Wharton and Ramløv, 1995). Inside the cyst, the nematode embryo develops into the first stage juvenile (J1) which molts within the egg into a second stage juvenile (J2).

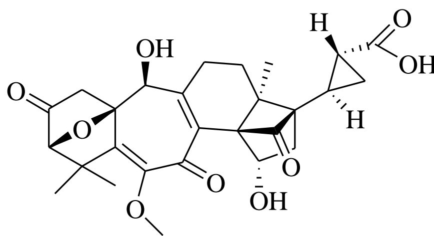
**2.2.3.2 Potato cyst nematode hatching factors (HFs)**

In the absence of a suitable host, PCN HFs can be directly applied in infested soils to induce hatching of juveniles which will eventually die since they are unable to feed thus inducing 'suicidal hatching' (Devine and Jones, 2000). This can be used as an environmentally friendly management strategy. Both natural and synthetic compounds have been identified as PCN HFs. Most of these HFs have been successfully used in the laboratory set-ups but field application is yet to be achieved.

#### 2.2.3.2.1 Natural hatching factors

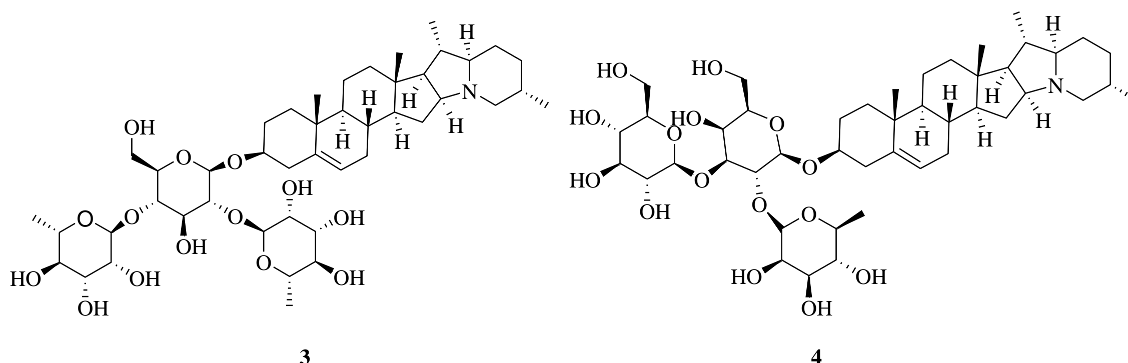
Previous studies have identified secondary metabolites exuded by host plant roots to play a key role in PCN hatching. These natural HFs have been isolated from root exudates of both potato and tomato (Devine *et al.*, 1996).

A secondary metabolite, solanoeclepin A ($C_{27}H_{30}O_9$) (**2**), which is a tetranortriterpene derived from gonanane has been identified as the main hatching factor (HF) for PCN and its structure elucidated by Schenk *et. al.,* (1999). Synthetic solanoeclepin A is able to stimulate hatching at concentrations as low as $1X10^{-10}$ g/ml of water (Tanino *et al.*, 2011). However, this hatching was only 65% of that induced by tomato root exudates, suggesting the existence of other co-HFs present in the exudates.




**2**

Glycoalkaloids α-solanine (**3**) and α-chaconine (**4**) have also been identified as *G. rostochiensis* HFs (Byrne *et al.*, 2001; Devine *et al.*, 1996). Both compounds were shown to be able to induce hatching with maximum hatch at a concentration of $1x10^{-3}$M (Devine *et al.*, 1996). The functionality of these three compounds could be attributed to their highly oxygenated structures that enabled them to bind to water molecules and be absorbed by the eggshell membrane stimulating J2 hatching (Torto *et al.*, 2018a).



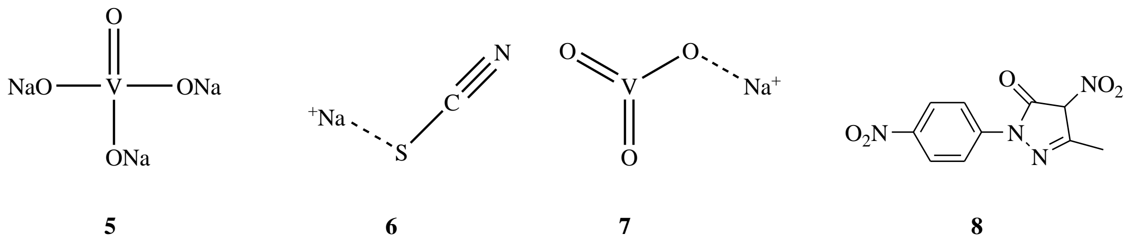
**3** **4**

Recently, Hoysted *et al.*, (2018), reported for the first time that simple sugars like glucose and fructose present in PRE can induce hatching of *G. pallida.* They further demonstrated that a blend of the two sugars stimulated even more hatching than when the compounds were used individually, though the blend was not better than the PRE.

A lot of work has been done on elucidation of natural HFs from the root exudates of host plants, but not on the identification of bioactive compounds involved. Sasaki-Crawley (2013) showed the hatching activity of *Solanum sisymbriifolium* even though the main hatching factor in potato, solanoeclepin A (**2**), was absent. This suggests the existence of other bioactive compounds working either individually or as part of a blend with the already identified glycoalkaloids and sugars. Testing of these compounds as blends has not yet been done. Although some studies have identified naturally occurring compounds that can stimulate cyst nematode hatch, the practical application for cyst nematode management is yet to be realized as their structural complexity currently makes them too expensive and a challenge to synthesize in sufficient amounts (e.g. solanoeclepin A).

#### 2.2.3.2.2 Synthetic hatching factors (HFs)

On different occasions, synthetic compounds have been demonstrated in *in-vitro* assays to stimulate PCN hatching. Some of these compounds include; sodium orthovanadate (**5**), sodium thiocyanate (**6**), sodium metavanadate (**7**) and picrolonic acid (**8**) (Byrne *et al.*, 2001; Devine *et al.*, 1996).



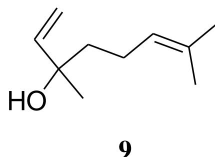

### 2.2.4 Potato cyst nematodes host finding

After hatching, PCN J2 rely on their lipid reserves as their only source of energy until they locate a host plant, penetrate the root tissues and establish the syncytium in the vascular cylinder. Under optimal conditions the infective life of a *G. rostochiensis* is 6-11 days. A 65% reduction in the reserves significantly reduces PCNs motility and infectivity (Robinson, 1986). In the rhizosphere, there exist multiple semio- and physico-chemical gradients around physiologically active growing plants, including volatile and non-volatile compounds, temperature, pH, or redox potential among others. Such chemical and tactile cues are deployed by soil-dwelling nematodes as primary signals for host plant detection, orientation, guidance and communication in the rhizosphere (Rasmann *et al.*, 2012).

The response of cyst nematodes to chemical stimuli is generally assumed to be perceived by the external sensilla, comprising the posterior and anterior amphids. The amphidal canal houses the neuronal dendrites that are bathed in secretions from the socket cells that may facilitate signal transduction. These secretions have been found to contain proteins that may play an important role in chemoreception (Masler and Perry, 2018). Chemical stimuli encounter these secretions and reach the receptors through diffusion. The amphids contain multiple sensory neurons and variable secretory materials, making them selective and sensitive signal transducers (Masler and Perry, 2018; Reynolds *et al.*, 2011; Robinson and Perry, 2006). For cyst nematodes, chemical disruption can be achieved by blocking the amphids, which are the nematodes' main chemoreception organs (Bird, 2004; Curtis, 2008), thus, can be exploited for their management.

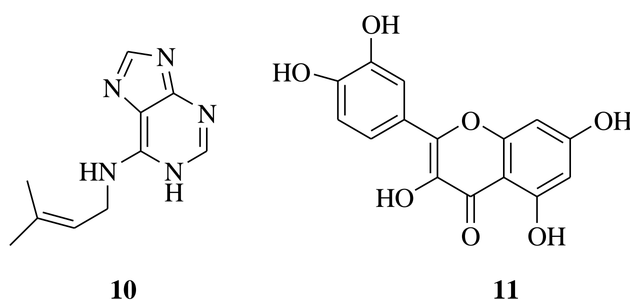
The primary stimuli for locomotion and host-seeking of cyst nematodes are semiochemicals produced by host plant roots. Behavioral studies have shown that most cyst nematodes are attracted to the roots or root-produced compounds from the host plant (Devine and Jones, 2003; Farnier *et al.*, 2012; Hu *et al.*, 2017; Papademetriou and Bone, 1983; Reynolds *et al.*, 2011; Spence *et al.*, 2008; Viglierchio, 1961; Wang *et al.*, 2018). Perry (2005) grouped the attractants into three categories i) long distance attractants, that enable location of the host plant root general area ii) short distance attractants, that direct J2 to a certain root and iii) local attractants, that guide J2 to the particular site of invasion. While host-specific compounds are most likely among the most important stimuli for cyst nematode chemoattraction, there are other factors that may help orient J2 to specific sites on the root. Such factors include sugars, pH, amino acids and $CO_2$ (Perry, 2005).

For PCN, a lot of work has been done on short range chemical cues from host plant root exudates that mediate host location. Devine and Jones, (2003) identified three classes of compounds, i.e., chemoattractants, chemostatics and chemorepellants present in PRE that influence PCN behavior. The exact identities of these compounds still remain unknown. Būda and Čepulytė-Rakauskienė, (2015) demonstrated PCN attraction to α-solanine (**3**). However, linalool (3,7- dimethylocta-1,6-dien-3-ol) (**9**), a root volatile from most solanaceous plants has been demonstrated as an attractant for PCN J2s (Būda and Čepulytė-Rakauskienė, 2015).

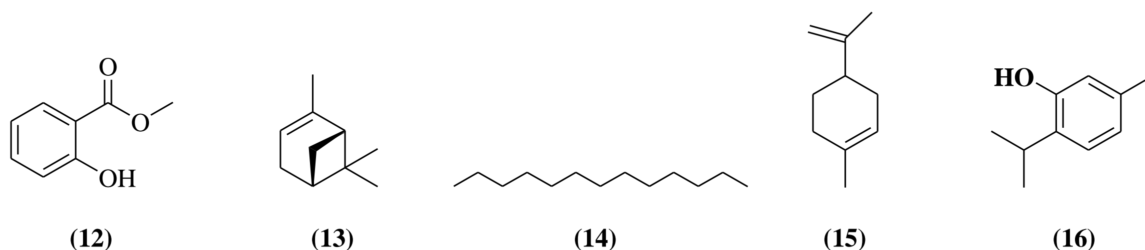



**9**

For other PPNS such as the RKN, *M. incognita* both long- and short-range attractants have been reported (Kihika *et al.*, 2017; Kirwa *et al.*, 2018; Murungi *et al.*, 2018). For example, Kirwa et al. (2018) demonstrated attraction of the root knot nematode *M. incoginta* to water soluble root exudates (short distance attractants) from tomato plants. Further fractionation of the exudates and bioassay guided chemical analysis identified a plant hormone, zeatin **(10),** and a flavonoid, quercetin **(11),** as significant RKN attractants to tomato plants. Another study also showed exudates from root tips of maize and green pea (*Pisum sativum* L.) resulted in a reduction of nematode infectivity by induction of a dormancy-like state (Jaffuel *et al.*, 2015; Zhao *et al.*, 2000)




Apart from the short range cues, RKNs are also attracted to plant volatiles. Kihika *et al.*, (2017) demonstrated RKNs attraction to volatile organic compounds produced from pepper roots. Methyl salicylate (**12**) was found to induce the highest positive chemotaxis compared to α-pinene (**13**) tridecane (**14**), and limonene (**15**). Murungi *et al.*, (2018) also identified methyl salicylate, 2-isopropyl-3-methoxypyrazine and tridecane as strong contributors of the attractiveness of tomato and spinach plants to RKNs. Also present in an accession AVDRC PP0237 of pepper, was a phenolic monoterpene, thymol (**16**), which elicited avoidance of the plant and showed toxicity at the highest dose tested.




### 2.2.5 Potato cyst nematodes damage to crops

Assessing nematode impact on a crop is difficult since the nematode damage is not as obvious as those caused by other pests and diseases (Nicol *et al.*, 2011). Above-ground symptoms of PCN infestation occurs as patches of stunted growth, yellowing of leaves and plant wilting. These symptoms only occur when infestations are very high and they

are similar to water and mineral deficiency, as PCN damages the ability of plants to uptake water and minerals (Holden, 2015). The poor growth of PCN infested plants leads to a reduction in both quality and quantity of yields resulting in huge production losses (Holden, 2015).

### 2.2.6 Potato cyst nematodes control measures

Several regulations and legislative control measures have been put in place in some countries that aim at preventing further spread and multiplication of these pests to non-damaging levels (EU, 2007). Also, several chemical and non-chemical measures have also been put in place to control PCN occurrence (Lambert and Bekal, 2002). Some of these control strategies are outlined below.

#### 2.2.6.1 Biological control

This includes the use of natural pathogens and predators of nematodes (Trifonova and Atanasov, 2011) found in nematode suppressive soil where they limit nematode damage to plants below the economic threshold. These predators include bacteria, mites, insects, protozoa, and fungi (Mankau, 1980). Microorganisms antagonistic to PPNs have been studied to identify suitable biological control strategies (Nyaku *et al.*, 2017; Toju and Tanaka, 2019). The activity of such microorganisms includes various modes of action including direct parasitization, competition for nutrients, induced systematic resistance, promoting plant health, and producing toxins, antibiotics and enzymes (Cronin *et al.*, 1997; Hackenberg *et al.*, 1999; Hasky-Guenther *et al.*, 1998; Reitz *et al.*, 2000; Tian *et al.*, 2007).

Some antagonistic fungus e.g., *Trichoderma spp* have been shown to be effective in controlling nematodes (Bokhari, 2009; Umamaheswari *et al.*, 2012). *Trichoderma asperellum* has been used commercially as a bio-nematicide and its mode of action includes directly competing with the nematodes for biological space in the plant and enhancing plant immune system and parasitizing the competing microbes. Biological control using nematophagous fungus *Paecilomyces* Sp. has also been used in integrated pest management with crop rotation to achieve a 89% PCN population decline (López-Lima *et al.*, 2013)

Some antagonistic bacterial isolates from a range of genera including *Providencia, Pseudomonas, Paracoccus* and *Serratia* have been shown to distort and disintegrate *G. rostochiensis* J2 (Back *et al.*, 2018; Riga *et al.*, 1996; Salinas-Castro *et al.*, 2016). Microorganisms can also produce compounds that are able to interfere with PCN life stages. For example, the fermentation of the soil hyphomycete fungus, *Myrothecium verrucaria,* has led to the commercialization of the biological-based nematicide DiTera®, which is effective for the control of *H. glycines* and *H. schachtii,* among other PPNs (Warrior *et al.*, 1999), and can irreversibly prevent hatching of PCN (Perry *et al.*, 2000). Other bacterial isolates such as *Agrobacterium radiobactor* have been shown to inhibit hatching of *G. pallida* (Hackenberg and Sikora, 1994; Ochola et al., 2020b) and *H. schachtii* (Oostendorp and Sikora, 1990). Chitinase producing bacteria, *Stenotrophomonas maltophilia* and *Chromobacterium* sp., inhibited *G. rostochiensis* hatch (Cronin *et al.*, 1997).

Microbial volatile organic compounds (VOC) have been shown to influence interactions within the rhizosphere. These volatiles can be nematicidal, anti-fungal, growth-promoting or inhibiting among others (Costa *et al.*, 2015; Gu *et al.*, 2007). Studies to date on the effect of microbial VOCs on PPN have largely concentrated on root-knot nematodes (RKN; *Meloidogyne* spp.), which limit both our understanding of the role of these compounds, as well as their potential as a management tool for cyst nematodes. Zhai *et al.*, (2018; 2019) showed that the VOCs cyclo(l-Pro–l-Leu) produced by *Pseudomonas putida* strain 1A00316 isolated from Antarctic soil was nematicidal and highly inhibited the hatch of the RKN *M. incognita.* A VOC from the same family, cyclo(d-Pro-l-Leu), has been isolated from *Bacillus amyloliquefaciens* Y1, with similar nematicidal characteristics (Jamal *et al.*, 2017). The range of effects of these compounds on cyst nematodes is yet to be investigated.

#### 2.2.6.2 Resistant cultivars

Specific resistance genes that interact with virulence genes of a pathogen leading to formation of a gene-for-gene relationship, often govern plant resistance. Potato cyst nematode, *G rostochienis,* and potato carrying H1 resistance gene have such a relationship (Bohlmann, 2015). The use of resistant cultivars is the most effective control measure because it is environmentally safe and an economical means of managing damaging PPNs (Barker and Koenning, 1998). Resistant cultivars are able to increase yield and reduce the number of cysts in the soil (Urek *et al.*, 2008). The mode of action of PCN resistant cultivars include feeding site degeneration, disabling the ability of

nematodes to obtain sufficient nutrients for formation of effective fertilized females, thus no reproduction (Haydock and Evans, 1998).

Although, use of resistant cultivars are effective, the main shortcoming lies in the ability of other nematodes to grow on resistant cultivars (Lambert and Bekal, 2002). For example, a study carried out in England and Wales showed that repeated use of a cultivar resistant to *G. rostochiensis* allows *G. pallida* species to reproduce and become the dominant species (Hockland, 2002). In countries like Kenya where PCN is a newly reported invasive species, resistant cultivars are also not easily available, or not widely accepted by farmers due to their longer cooking time or their taste, compared to variety Shangi. Resistance to PCN is both species- and pathotype- specific and having several PCN pathotypes for each species; no cultivar is able to control both species at the same time.

Tolerant varieties are also an alternative that can reduce yield losses. Unfortunately, tolerant varieties result in increased cyst populations in the soil, though the losses caused by the nematodes are reduced.

#### 2.2.6.3 Crop rotation

This measure involves growing non-host or antagonistic plants for a period to allow cyst populations in the soil to decline. PCN having a narrow range of hosts (selected solanaceous crops) could imply that it can effectively be managed by crop rotation (Perry and Moens, 2011). However, this is not the case because of longevity of the nematode in

the soil and spontaneous hatching of PCN eggs in the absence of a host is as low as 20-40% per annum (p.a.) for *G. rostochiensis* and 10-30% p.a. for *G. pallida* (Sparkes, 2016). Therefore, in heavily infested soils, it can take several years to reduce the population to non-damaging levels (Kerry *et al.*, 2009). Hancock (1988) suggested 7-8 years of rotation is necessary to reduce populations to that before the last potato crop.

The mode of action of the non-host plants used in the rotation differs. For example, the litchi tomato can stimulate PCN egg hatching but does not allow the nematode to fully develop and reproduce, thus reducing the nematode densities in the soil. Root exudates from some non-host plants are believed to contain toxic compounds that are released into the soil where they have nematicidal activities. Antagonistic plants such as asparagus produce asparagusic acid that is toxic to several nematodes and inhibits juvenile emergence from the cyst.

#### 2.2.6.4 Trap crops

Stimulation of egg hatching in the absence of a host is one way to eradicate cyst nematodes from infested soil. Treatment of soil with HFs in the absence of a suitable host could induce a 'suicide hatching' since the juveniles will die due to lack of a suitable host (Sparkes, 2016).

Trap crops uses this principle to stimulate hatching of the eggs and 'trapping' the nematode in the plant where they cannot reproduce. Early uprooting of some host plants can also be used to limit nematode reproduction by destroying the plants before the

females mature. This requires very careful timing not to allow the female to mature but to allow establishment of reasonably sized roots (Hockland, 2002).

Trap crops have been shown to significantly reduce the number of cysts in the soil (Scholte, 2000a; Timmermans *et al.*, 2007). Among the trap crops for PCN is *S. sisymbriifolium* (Lamarck) which has shown hatch simulations similar to a potato crop with complete resistance (no progeny cysts) (Scholte, 2000a). Lamarck is completely resistant to both *G. rostochiensis* and *G. pallida* and is currently used in the Netherlands and the UK (Scholte, 2000a; Timmermans *et al.*, 2007).

The mode of action of Lamarck includes a mechanism that prevents the development of second stage juvenilles in the plant. Two *Solanum nigrum* complex species have also shown full resistance to *G. rostochiensis* and high level of resistance to *G. pallida* in addition to performning very well in temprate regions in Ductch fields (Scholte, 2000b). Interestingly, some non-solanacea plants, such as *Oxalis tuberosa* (Oxalidaceae) and lupine (*Lupinus mutabilis* (Fabaceae), have also demonstrated efficacy as PCN trap crops (Franco *et al.*, 1999). Ongoing studies in Kenya have also demonstrated the effectiveness of two African nightshade species, i.e. *S. vilosum* and *S. scabrum,* as potential trap crops for PCN. These two indigenous African vegetables showed an 80% decline in cyst populations after three seasons (Chitambo *et al.*, 2019).

#### 2.2.6.5 Organic amendments

Most organic soil amendments are meant to improve soil structure and increase its organic content, enabling it to hold more moisture and nutrients. Their success in PPN management is attributed to a combination of different mechanisms, which include production of bioactive compounds like nematode repellents, hatching inhibitors (HIs) or nematicides into the rhizosphere, increase or introduction of antagonistic microorganisms into the soil and boosting defense mechanisms of the plants. Organic amendments are applied by mixing of soil with dried plant parts in cake or powder form before or after planting.

Bioactive components of organic amendments can be extracted from the plant material by soil moisture or nematode secretions, which facilitate their slow release into the soil over time. Microbial degradation of the plant parts could lead to changes in bioactive components exposed to the nematodes, which minimize their resistance. Plant tissues of species of *Brassicaceae* family like *Brassica juncea* contain glucosinolates and these degraded into isothiocyanates which are used for their nematicidal effects in biofumigation programs (Torto *et al.*, 2018b; Wu *et al.*, 2011). Allyl isothiocyanates have also been identified as PCN hatching inhibitor (Ellenby, 1945; Wood *et al.*, 2017).
Plant parts from the *Brassicaceae*, *Meliaceae*, *Asteraceae* families have been used as organic amendments in the control of PCN (Ellenby, 1945; Fourie *et al.*, 2016; Valdes *et al.*, 2012; Wu *et al.*, 2011). Although organic amendments are environmentally friendly, some studies suggest that large quantities may be required per unit area to make them work effectively (Moens *et al.*, 2009).

#### 2.2.6.6 Chemical control

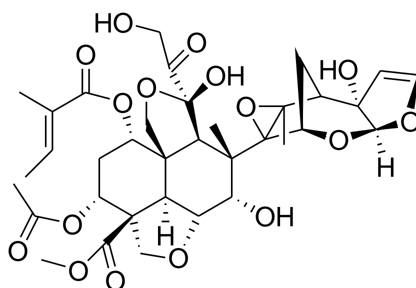
Chemical control involves the use of nematicides to either kill (true nematicides) or paralyze nematodes (nematistats). Nematistats paralyze nematodes for a specific amount of time to allow depletion of their lipid reserves. This is the most used management method for PPNs (Haydock *et al.*, 2006; Khalil, 2013).

Broadly, nematicides can be classified into soil fumigants and non-fumigants (Schomaker and Been, 2006). The mode of action of both non-fumigant organophosphate and granular carbamate nematicides is to disrupt the feeding, metabolism and movement of the juveniles. These pesticides are not very effective in PCN control because they may decompose before all the juveniles, hatch (Hockland, 2002). Soil fumigant nematicides are the most effective, although most of them have been banned due to environmental concerns (Lambert and Bekal, 2002). The use of the other fumigants, e.g. diazomet, 1,3-dichloropropene and chloropicrin, are heavily regulated (Lambert and Bekal, 2002).

The use of synthetic chemical nematicides has had various drawbacks that include adverse environmental impact, resistance development and toxicity to non-target organisms (Khalil, 2013). Theses drawbacks have led to an increased interest to develop more environmentally friendly and more target specific nematicides from natural sources (biopesticides) (Khalil, 2013; Trifonova and Atanasov, 2011).

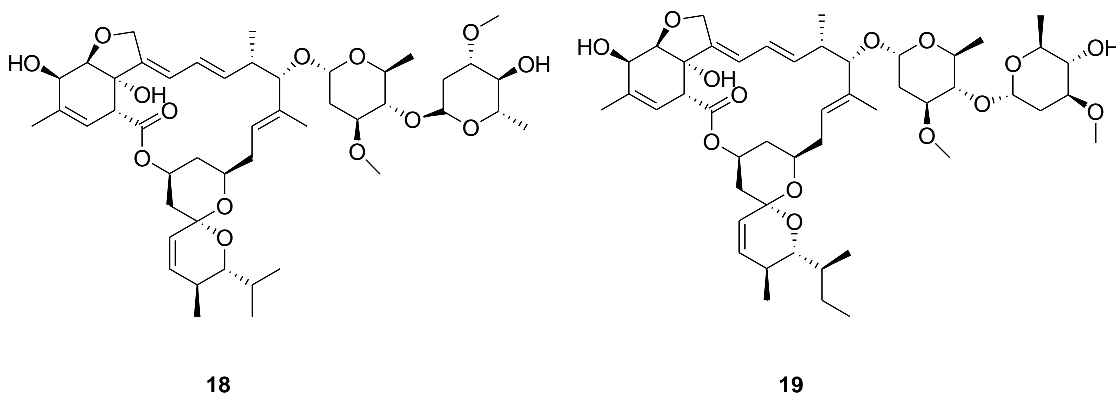
*Azadirachta indica* or the neem tree, is one of the plants that produce various products effective in protecting plants against insects and PPNs, and improves plant health when used as root-dips and seed treatments (Akhtar, 2000; Khalil, 2013). Neem products are able to reduce egg hatch and mobility of the juveniles (Kumar and Parmar, 1998).

One of the compounds isolated from the kernels of neem seeds is a limonoid triterpenoid, azadirachtin (**17**) (Chitwood, 2002). Azadirachtin has been used as a natural and eco-friendly pesticide because of its safety to humans and the microbial community and has zero environmental persistence due to its rapid degradation in water in the presence of light (Chitwood, 2002).




**17**

Natural fermentation of a soil bacterium *Streptomyces avermitili* produces abamectin which is a blend of two avermectin compounds (80% avermectin B1a (**18**) and 20% avermectin B2b (**19**) (Cao *et al.*, 2016) with similar biological and toxicological profiles (Khalil, 2013). Apart from the application as a control agent for insects and mites, abamectin is also used to protect plants in the initial growing phase from attack by PPNs (Dybas, 1989; Khalil, 2013). It works by binding to the glutamate receptors in the muscle and nerve cells of the PPN causing their motor paralysis (Cao *et al.*, 2016).




Abamectin has found great advantage over other nematicides due to its strong activity against a broad spectrum of nematodes, low toxicity to non-target beneficial arthropods, moderate environmental persistence, low human toxicity, no underground water contamination and residue persistence (Khalil, 2013). However, its efficiency is greatly compromised by its poor mobility in the soil due to its low water solubility and high tendency to bind to organic carbon content in the soil (Krogh *et al.*, 2008). This leaves a gap for the development and deployment of an alternative that will overcome this disadvantage.

### 2.7 'Wrap and Plant' ('W&P') technology

'Wrap and Plant' technology is an innovation developed in North Carolina State University in the United States of America (USA) for crop protection against soil pathogens and pests. This technology uses a lignocellulosic matrix made from banana fibers (banana paper) impregnated with abamectin (**18** and **19**) as a seed wrap (Plate 2.1). The paper is made through a mechanical process where slurry of the fibers is mechanically beaten together with water to break the fibers into shorter sizes, the water is removed, and the fibers are pressed to produce paper. The paper produced has a high

burst strength that allows it to stay intact in the soil, but at the same time can allow roots to penetrate through it (Cao *et al.*, 2016). Apart from the physical properties of the paper, it is also optimized to give a slow and sustainable release of the active ingredient abamectin by the high lignin content of the paper (Cao *et al.*, 2016). The use of banana paper impregnated with abamectin is able to overcome the main drawback of abamectin in that it offers a more targeted delivery of the nematicide thus minimizing interaction with the soil matrix (Cao *et al*., 2016).

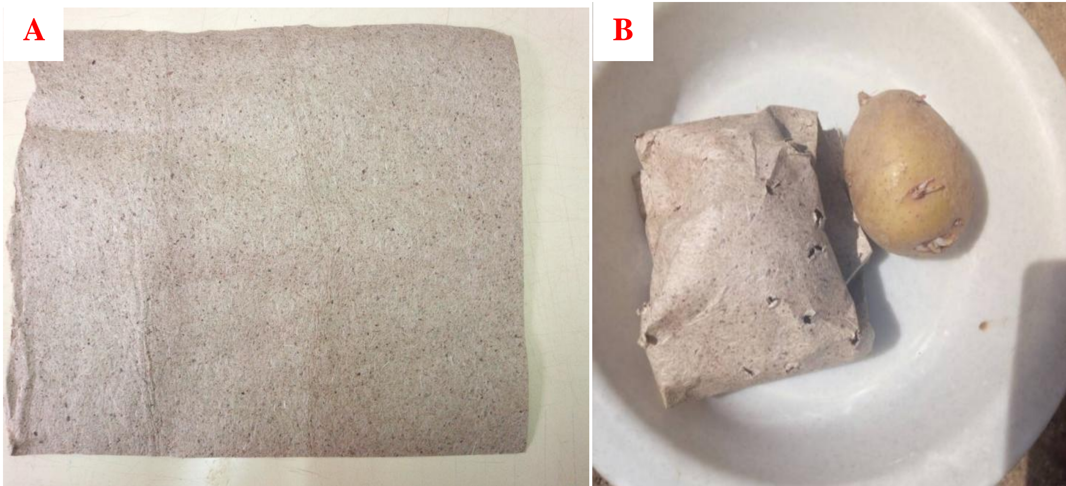



**Plate 2.1:** Banana fiber paper (A) and potato tuber wrapped with the banana fiber paper before planting (B) (Photo by Mwaura O. 2017)

Field trials were carried out on potato farms in Nyandarua County, Kenya, to evaluate the effectiveness of banana fiber paper in the management of PCN. The potato seeds wrapped with the treated banana paper before planting were more efficient in controlling PCN attack relative to the control (farmers' practice) where no paper was employed. It showed a 4-fold increase in yields and an 85% reduction in cyst populations compared to the farmers' practice. The treated banana paper also showed a 2-fold increase in yields compared to the nematicide, abamectin (Tervigo®) alone. The cyst decline in the treated

paper was also ~2-fold relative to the abamectin sprayed plants (Cortada *et al.*, 2018). Trials were conducted in two different sites in Nyandarua County in two consecutive potato growing seasons (2016-2017).

When the untreated banana paper was used to wrap potato seeds, there was also a 3-fold increment in yield and a 43% decline in cyst populations compared to the control where no paper was employed. The results suggested that apart from abamectin impregnated on the paper, the paper alone could also offer some protection to the plant. Although the effectiveness of the banana paper to manage PCN attacks on potato plants has been well established, the underlying mechanism that enables it to do so still remains unknown. This study sought to explore the mechanism by which the banana paper reduces PCN attack on potato plants.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Preparation of experimental plant materials

Potato (*S. tuberosum*) cultivar 'Shangi' commonly grown in Kenya, was used for the experimental purposes. The potato seeds were planted in 2 L plastic pots (13 cm base diameter, 17 cm top diameter and 15 cm deep, Kenpoly Manufacturers Limited, Nairobi, Kenya) (Plate 3.1) with perforated bottom filled with sterilized sand, autoclaved for 40 min at 121 ˚C (Astell Scientific autoclave, UK). The plants were grown in a screenhouse at the International Centre of Insect Physiology and Ecology (*icipe*) Duduville Campus, Nairobi, Kenya (1º 16' 60'' S; 3 6 º 4 9' 0 '' E). The temperature was maintained at 23 ± 3 °C and relative humidity 60-70%. Watering of the plants was done twice a week using nutrient solution prepared as outlined in section 3.2. Three to six weeks old plants were used in the study.

**Plate 3.1:** Two weeks old potato plants of variety 'Shangi' planted in pots in a screenhouse

## 3.2 Preparation of plant nutrient solution

Nutrient solution to supplement micro- and macro-nutrients to the plants was prepared as described by Lambert *et al.* (1992). The stock solution of $KNO_3$ (184 g/L), $MgSO_4.7H_2O$ (399 g/L) and $Ca(NO_3)_2.4H_2O$ (653 g/L) were prepared and autoclaved at 121°C. One liter solutions of macronutrients; $NH_4H_2PO_4$ (108 g) and $FeSO_4$ $7H_2O$ (10 g) plus 72 ml of 500 mM EDTA maintained at pH 8.0 was separately prepared. Micronutrients $CuSO_4.5H_2O$ (0.1 g), $MnCl_2.4H_2O$ (1.81 g), $H_3BO_3$ (2.83 g), $ZnSO_4.5H_2O$ (0.22 g) and $H_2MoO_4.H_2O$ (0.02 g) were prepared in 1 L solution and filter-sterilized using Whatman No. 1 filter paper (Macherey-Nagel, Oensingen, Switzerland). During watering of the plants, the nutrient solution was formulated by mixing 25 ml of each the prepared micro and macronutrients and 75 ml of $KNO_3$ with 50 L of distilled water in a plastic container.

## 3.3 Procedure for collecting PCN, *G. rostochiensis* population

Potato cyst nematodes (PCN) population was obtained from soil samples of a freshly harvested farm in Nyandarua County, Kenya (0.1804° S, 36.5230° E) previously identified to be infested with *G. rostochiensis*. The soil was dried and cysts were extracted by a floatation technique that uses a Fenwick can (Plate 3.2) as described in EPPO (2013).

**Plate 3.2:** Fenwick can

The collected cysts were dried in a milk filter paper before handpicking with the aid of a stereomicroscope (LEICA M125, Singapore) and sorting based on the color. Freshly formed cysts which were light brown in color were used for the hatching, chemotaxis and PCN development experiments.

### 3.4 Chemicals and Reagents

LC-MS grade methanol (LC-MS LiChrosolv®, Merck (≥ 99.97%), formic acid (98-100%), water (LC-MS Chromasolv), trans-zeatin (≥97%), solasodine (≥95%), tomatidine hydrochloride (≥85%), α-solanine (≥95%), solanidine (≥95%), phenylalanine (≥ 98%), tyrosine (≥ 98%), tryptophan (≥95%), methyl dihydrojasmonate (≥96%) and DMSO (≥99.9%) were purchased from Sigma-Aldrich (St. Louis, MO) and α-chaconine (≥95%) (PhytoLab, Germany).

### 3.5 Preparation of potato root exudates

Potato root exudates (PRE) were collected using the dipping method as described by Farnier *et al.* (2012) from potato cv 'Shangi'. The plants were gently removed from the

soil and the roots washed in tap water to remove sand particles before rinsing in distilled water. Batches of 5 plants were collected together to constitute one replicate and the roots immersed in 1 L of distilled water in 2 L plastic containers (Kenpoly Manufactures Limited, Nairobi, Kenya) as shown in plate 3.3. The container was wrapped with aluminum foil to exclude sunlight and the setup left for 24 h. For the hatching and chemotaxis assays, the fresh raw exudates were used but for the chemical analysis, the exudates were freeze-dried. Three to six weeks old plants were used in these experiments.

**Plate 3.3:** Potato root exudates collection

### 3.6 Determination of PCN, *G. rostochiensis* hatching response in the presence of banana fiber paper

Hatching assays were carried out to evaluate the effects of different treatments on PCN hatching. The treatments were applied as shown in table 3.1:

**Table 3.1:** Summary of hatching bioassay experimental treatments

| Paper presence | Treatment | Test solution | Treatment combination |
|---|---|---|---|
| With Paper | Untreated banana paper (UBP) | Water | UBP+Water |
| | | PRE | UBP+ Exudates |
| | Treated banana paper (TBP) | Water | TBP+Water |
| | | PRE | TBP+ Exudates |
| Without paper | Nothing | Water | Water alone |
| | | PRE | Exudates alone |

Test solutions comprised of PRE (a known PCN hatching stimulant acting as a positive control) and distilled water (spontaneous hatching of PCN) were applied to the treatments as illustrated in figure 3. 1.

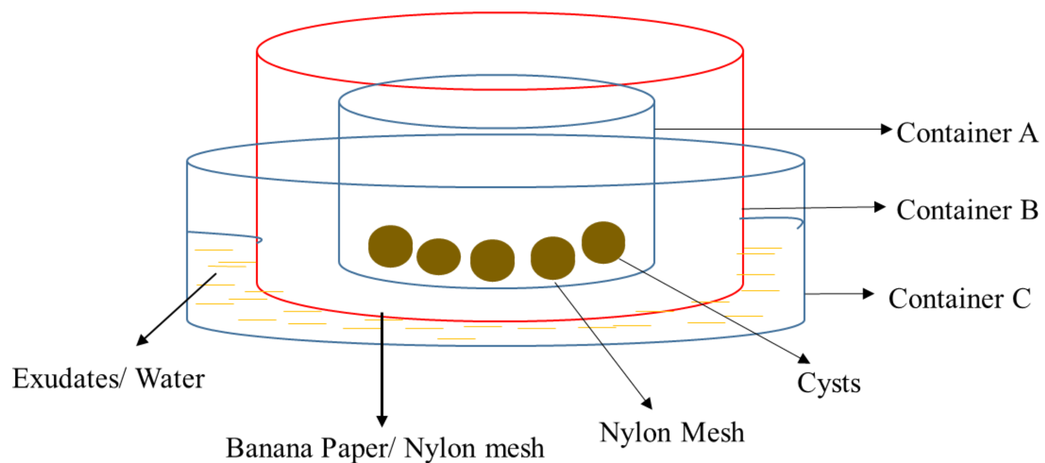



**Figure 3.1:** Schematic diagram demonstrating bioassay setup for hatching of PCN

Five cysts were placed in the set up shown in figure 3.1. For the paper treatments, container B was lined with the banana paper at the bottom, and for the control a nylon mesh was used to line the container instead. The set up was placed in a 6 well culture plate (Falcon, Becton Dickinson, labware) represented as container C where the test solution was added. The set up was left for 8 weeks and the number of J2s emerging from the cysts in each treatment counted and recorded weekly before replenishing the test solution. Exudates from 3-6 -week-old plants were separately tested in this experiment and replicated four times.

To check the effects of the treatments on egg viability, the cysts from the hatching assays after 8 weeks were cleaned using distilled water and subjected to a viability test. This was done using Nile blue A stain (Sigma-Aldrich, India) as described by Ogiga and Estey, (1974). Two ml of 0.05% of the stain was added to cysts in a 24 well culture plate. The set-up was left for 48 h scoring of viable (non-stained) and non-viable (stained) eggs were done with the aid of a stereomicroscope (LEICA M125, Singapore) under magnification of x40.

### 3.7 Determination of chemotactic responses of *G. rostochiensis* second stage juveniles (J2) to PRE in the presence of banana fiber paper

Cysts were placed in freshly collected PRE in a multiwall plate at 20 ± 1 °C in the dark for 3 weeks to induce hatching. The first emergence of J2s was seen in 7-12 days. The J2s were harvested daily by sieving using a 20µm sieve (ASTM E -11 standard No 635) to remove the exudates and the J2s recovered from the sieve into distilled water. Less than 24 h-old juveniles were used in the chemotaxis assays.

Chemotaxis bioassays were carried out according to the method described by Kirwa *et al.* (2018) with some modifications to monitor the response of hatched J2s to PREs in the presence and absence of banana paper. The set up described in figure 3.2 was filled with 5 g of sand in each chamber. Sand in chamber A and B was mixed with 1 ml of freshly collected PRE (less than 24 h. old) and distilled water (control) respectively as illustrated in figure 3. 2.

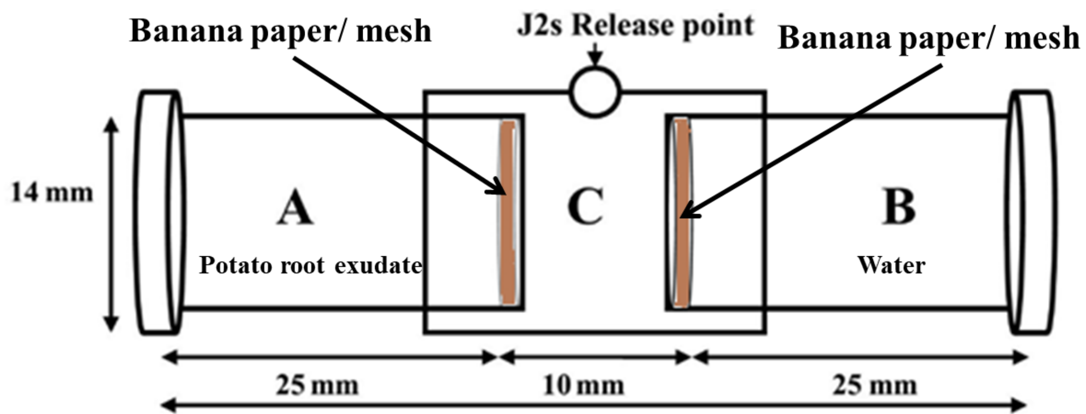



**Figure 3.2:** Schematic diagram of dual choice *in-vitro* chemotaxis assay set up.

The banana paper (untreated and treated with abamectin) was used to partition chamber A and C and chamber B and C for the paper treatments. For the control (no paper), a nylon mesh was used in the partitioning in place of the banana paper. One ml of water containing 200 hatched J2s was introduced in chamber C. This setup was left for 48 h. after which J2's present in the three chambers were recovered separately from the sand. This was replicated 3 times for each experiment and for 5-week-old plants (where we got optimum activity), the experiment was repeated five times.

The recovery was done by re-suspension of the sand in 100 ml of water and sieving using 20 µm and 38 µm sieves (ASTM E -11). J2s were recovered from the 20 µm sieve by suspension in 30 ml of water in 50 ml falcon tubes. The J2s were then picked using a 2 ml plastic bulb pasture pipette and placed in a counting dish where the nematodes were scored under a stereomicroscope. Nematodes present in chamber A were scored as positive responders to stimuli, in chamber B were negative responders and the ones in chamber C were non-responders.

### 3.8 Determination of the effect of banana fiber paper on *G. rostochiensis* development

'Shangi' potato variety was planted in previously autoclaved sand using 2 L plastic pots as described in section 3.1 with application of three treatments, these being i) potatoes wrapped with abamectin impregnated banana paper 'treated banana paper', ii) potatoes wrapped with untreated banana paper, and lastly iii) unwrapped potatoes, no paper treatment (control). The pots were inoculated with 20 cysts at planting and left to grow for 8 weeks in a screenhouse at 23 ± 3 °C. Plant nutrient solution described in section 3.1 was added during watering that was done three times in a week. A total of 72 pots were planted, 24 plants per treatment. Six replicates of each treatment were randomly selected and assessed after every two weeks for the different stages in nematode development. Counts were taken after every 2 weeks over a span of 8 weeks. This experiment was carried out two times.

To access the different developmental stages of PCN in the roots, the roots were stained according to a method by Bybd Jr *et al.* (1983). The roots were cut from the rest of the plant and thoroughly washed to remove soil debris before weighing (fresh mass). The washed roots were chopped into 1-2 cm segments and placed in 1.5% sodium hypochlorite solution (Jik bleach; Orbit chemical industries, Kenya) with occasional agitation for 4 min. Rinsing was then done in tap water followed by distilled water. The clean roots were placed in 30 ml of water to which 1 ml of acid fuchsine (3.5g in 25% acetic acid) (BDH, England) was added (Plate 3.4).

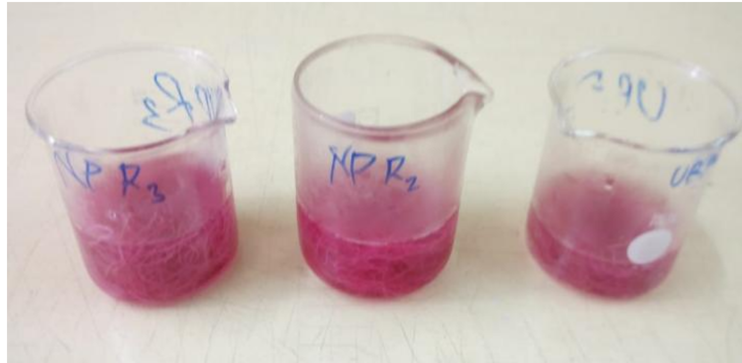



**Plate 3.4:** Potato roots stained in acid fuchsine stain

The solution was heated to boiling in a hotplate (Stuart® UC152), cooled and excess stain removed with running water. De-staining of the roots was done by immersing the roots in 20-30 ml of glycerin acidified with a few drops of 5N hydrochloric acid, heating and cooling. Root segments were then pressed between two microscope slides and scoring of the different nematode development stages done with the aid of a stereomicroscope under 40 x magnification.

## 3.9 Determination of the interaction between PRE and banana fiber paper

### 3.9.1 Determination of the chemical composition of PRE

To determine the chemical compositions of PRE, 10 mg of the freeze-dried root exudates as weighed in an Eppendorf tube and dissolved in 1 ml of 30% methanol. The sample was vortexed for 10 sec, sonicated for 30 min before centrifuging for 10 min at 14000 rpm. The supernatant was diluted to 1 mg/ml and transferred into a sample vial and 0.1 µl analyzed using an Ultra-Performance Liquid Chromatography coupled to a triple quadrupole tandem mass spectrometry (UPLC-QqQ-MS/MS). Chromatographic separation was performed on a ACQUITY UPLC I-class system (Waters Corp., Milford, 151 MA) fitted with an ACQUITY UPLC BEH C18 column (2.1 × 150 mm, 1.7 µm particle size; Waters Corp., Wexford, Ireland), that was heated to 45 °C. The autosampler tray was cooled to 5 °C. The mobile phase comprised of water acidified with 0.01% formic acid (solvent A) and methanol (solvent B) and followed a gradient system. The gradient system used was 0-2 min, 5% B, 2-4 min, 40% B, 4-7 min, 40% B, 7-8.5 min 60% B, 8.5-10 min 60% B, 10-15 min, 80% B, 15-19 80% B, 19-20.5 min, 100% B, 20.5-23 min, 100% B, 23-24min 95% B, 24-26 min, 95% B. The flow rate was held constant at 0.2 ml/min. The gradient system used comprised of 0-2 min, 5% B, 2-4 min, 40%B, 4-7min, 40%B, 7-8.5 min 60%B, 8.5-10 min 60%B, 10-15 min, 80%B, 15-19 80%B, 19-20.5 min, 100% B, 20.5-23 min, 100%B, 23-24min 95%B, 24-26 min, 95%B. The solvent flow rate was kept constant at 0.2 mlmin$^{-1}$.

The UPLC was interfaced with an electrospray ionization (ESI) Waters Xevo TQ-S operated in full scan MS in both positive and negative ionization modes. Data were

acquired over the m/z range 100–2000 with a capillary voltage of 0.5 kV, sampling cone voltage of 30 V, source temperature 150 °C and desolvation temperature of 120 °C. The nitrogen desolvation flow rate was 600 L/h. Data was acquired using MassLynx version 4.1 SCN 712 (Waters). Potential assignments of compounds were determined after the generation of the mass spectrum for each peak, establishing the molecular ion peaks using adducts, common fragments, literature and where available, confirmed with authentic samples through co-injections. All the samples were analyzed in triplicate, with each replicate collected from different batches of plants.

### 3.9.2 Determination of the effect of selected authentic compounds from PRE compounds on PCN hatching

Ten synthetic standards: steroidal glycoalkaloids α-solanine and α-chaconine; the steroidal alkaloids solanidine, solasodine, and tomatidine hydrochloride; phytohormones, zeatin and methyl dihydrojasmonate; and amino acids, tryptophan, tyrosine and phenylalanine were used to evaluate their effect on PCN hatching. Even though not identified in PRE, steroidal alkaloid tomatidine and solasodine were also tested due to their structural similarity to solanidine. PCN *in-vitro* hatching bioassays were conducted according to the protocol used by Twomey *et al*. (1995). A stock of 1 mg/ml in 1% DMSO of the standards was prepared and diluted to five concentrations: 0.2, 0.4, 0.6, 0.8 and 1 µg/ml. A 200 µl aliquot of each test solution (standards) was added to each well of a Linbro® 96 multi-well sterile plate containing five cysts per well, pre-soaked in water for 5 days (Twomey *et al*., 1995). Each concentration was tested in six replicates. Potato root exudate was used as the positive control.

The 96-well plate was covered and placed in the dark at 20 ± 3°C. The emerging J2 were counted weekly and the test solutions replenished following each count. This was done for 5 weeks after which the remaining eggs within each cyst were assessed for viability using Nile blue A stain (5-aminobenzo[a]phenoxazin-9-ylidene)-diethylazanium sulfate - Sigma-Aldrich, India) and counts of the viable eggs done according to Ogiga and Estey (1974). Hatching activity for each experimental treatment was expressed as cumulative percentage (%) of hatched J2 recovered from the wells (emerged J2s) and the encysted hatched J2 recovered from the cysts. The total number of viable eggs was the sum of hatched J2 plus the viable eggs recovered from the cyst.

### 3.9.3 Determination of chemical composition of PRE after exposure to banana paper

To evaluate the chemical composition of PRE after exposure to banana paper an *in-vitro* experiment was carried out. One milligram of freeze-dried PRE was dissolved in 1 ml of 3:7 v/v blend of methanol:water. Fifty milligrams of banana paper (treated and untreated) was immersed in the sample and the set-up left for 24 h. The banana paper was removed and the remaining solution centrifuged for 10 min to remove any remaining particles and the remaining PRE analyzed on a LC-QqQ-MS/MS as outlined in section 3.7. This experiment was carried out using exudates from 3-6-week-old plants separately and replicated thrice.

### 3.9.4 Determination of the chemical composition of banana paper after exposure to PRE

Constituents of banana paper after exposure to PRE was determined by exposing 2g of banana paper (treated and untreated) to 30 ml of 1mg/ml PRE for 24 h. as described in section 3.6. The exposed paper was dried and then extracted using 30 ml of 5% methanol in a sonicator bath at room temperature for 30 min. This extract was freeze-dried and 1 mg of the dried extract reconstituted in 1 mL of 30% methanol. The sample was centrifuged (Spectrafuge 16M, Edison NJ, USA) at 14000g for 10 min. and analyzed by UPLC-QqQ-MS/MS as outlined in section 3.6 above to determine the chemical composition of the banana paper after exposure to PRE.

### 3.9.5 Determination of the chemical composition of PRE after exposure to pure cellulose

To evaluate the chemical composition of PRE after exposure to cellulose, an *in-vitro* experiment was carried out. 1mg of freeze-dried PRE was dissolved in 1 ml of methanol: water (3:7 v/v). Fifty milligrams of cellulose was immersed in the sample and the set-up left for 24 h. The set-up was centrifuged as illustrated in section 3.7.3 to allow the pure cellulose to settle and the supernatant collected and analyzed on a UPLC-QqQ-MS/MS as demonstrated in section 3.7. This experiment was carried out using exudates from 5-week-old plants and replicated three times.

### 3.10 Data Analyses

The number of emerging juveniles and non-viable eggs from the hatching bioassays were log transformed and subjected to analysis of deviance after fitting a linear mixed model with repeated measures and week taken as a random effect. The mean interactions of the treatments were analyzed using least squares means.

The number of responding J2s from the dual choice assays was analyzed using the proportionality test to check the effect of the treatments on the attraction potential of the stimulus. The differences across the treatments were determined for each of the three chambers separately including stimulus, control and the release chamber. The counts of the J2 recovered from the stimulus chamber and the release chamber were fitted in a generalized linear model (GLM) with a negative binomial distribution, and those recovered from the control chamber were fitted in a zero-inflated model with a negative binomial distribution. A pairwise comparison of the means was performed when there were significant differences in the means.

The different stages of nematode development were represented as a mean of 6 replicates and subjected to ANOVA after fitting in a generalized linear model with a negative binomial distribution. The means were adjusted using least-square means (LSM) and separated using Tukey's honest significance test. Data from the two experiments were pooled and analyzed to check the effect of the treatments on the development of PCN. The analysis was done for each week and at each developmental stage separately. The data was fitted in a generalized linear model (glm) with a negative binomial distribution

with the exception of J2 counts at week 2 which was fitted in a zero-inflated model with negative binomial distribution and the females count at week 4 which was fitted in the zero-hurdle model, to address both dispersion and zeros in the data. All the models took experiment and treatment as covariate.

For the effect of synthetic compounds on PCN hatching was expressed as a hatching index (HI) that was calculated according to the formula:

$$HI=(TH-CH)/ (TH + CH) \times 100$$

where, TH is the number of J2 hatching stimulated by a test compound and CH is the number of J2 stimulated by the positive control, potato root exudate. A positive value in the hatching index indicates that a higher proportion of the J2 hatched on stimulation by the compounds, compared to the control, whereas a negative value indicates the opposite effect. Plots of hatching index were made separately for the J2 that emerged from the cyst and those that remained encysted. The data on the hatched J2 were analyzed using a generalized linear model with a binomial distribution. Using the control potato root exudate as a reference category, the odds ratios (ORs), a measure of the likelihood that PCN responded to the other treatments instead of the control were estimated including Confidence Interval (CI) and corresponding *p*-values. With OR for the control set at 1, values above this indicates better hatching response and values below underperformance of the treatments, relative to the positive control. Differences between the compounds were evaluated using a test of proportions.

R-statistical program version 2.11.0 software was used to perform the statistical analyses, and all tests were performed at 5% significance level.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 Hatching response of potato cyst nematodes (PCN) in presence of banana fiber paper

The hatching response of PCN in the presence of banana paper was evaluated using two test solutions, water and PRE. The PRE alone treatment had the highest number of emerging J2's in the hatching assay after 8 weeks. This was observed in all the four experiments with PRE from 3-6 weeks old potato plants, as shown in Figure 4.1.

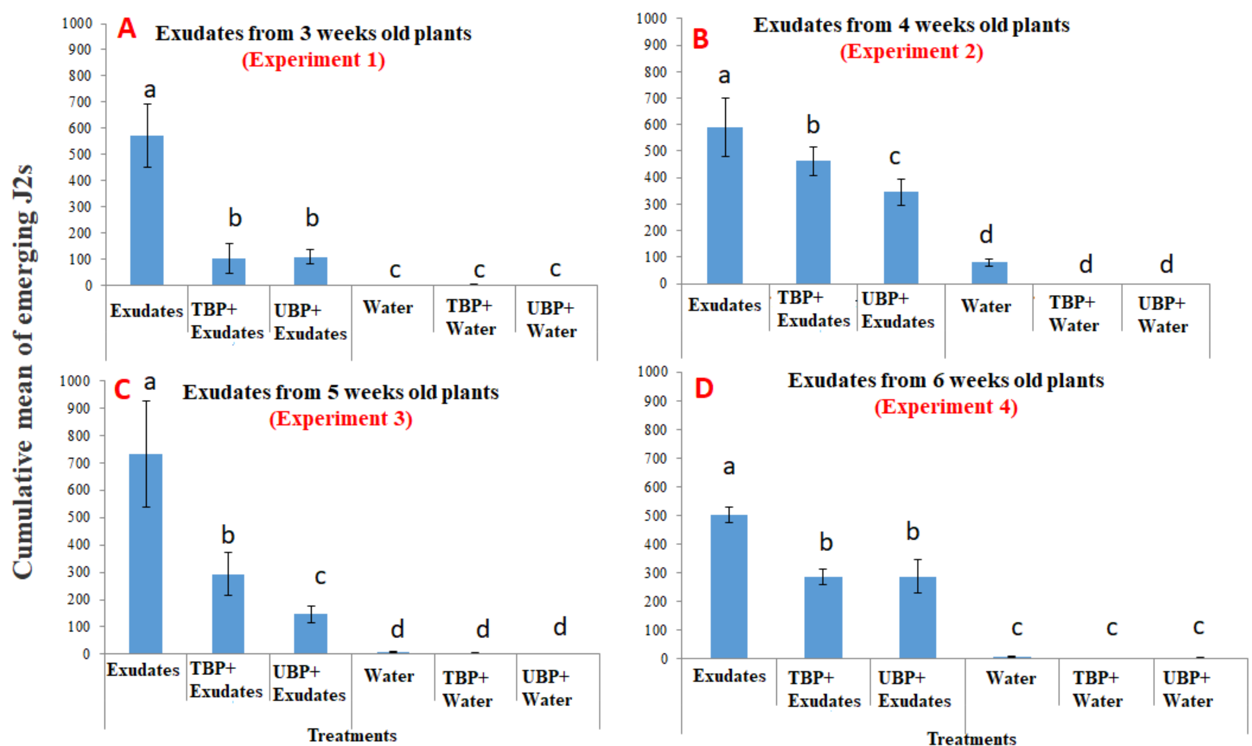



**Figure 4.1:** Hatching response of PCN J2s in the presence of different treatments after 8 weeks of exposure- (**A**): Hatching response to PRE from 3-weeks old plants; (**B**): Hatching response to PRE from 4-weeks old plants; (**C**) Hatching response to PRE from 5-weeks old plants; **(D)** Hatching response to PRE from 6-weeks old plants.

Comparison of the number of J2 hatched in the six treatments was based on hatching using PRE from 5-weeks-old plants, which stimulated the highest number of J2 hatching. Using exudates from 5-week-old plants, there was a significant difference between the treatments ($F_{(4, 15)} = 42.844$, $P < 0.001$). Exudates alone had the highest number of hatched J2s compared to the other treatments, i.e., 2.5 and 5.2-fold the number of J2s hatched in TBP+ exudates and UBP+ exudates respectively. The treatments with water as the test solution (water alone, TBP + water, UBP + water) gave the lowest number of hatched J2s and no significant difference was found between them. This trend was also observed in experiments 1, 2 and 4 but to varying degrees, as shown in Figure 4.1 A, B and D, suggesting that the age of the plant had an effect on the number of hatching J2s but not the overall hatching pattern in the presence of the banana paper.

After 8 weeks of exposure using PRE from 4- and 5-week-old plants, there was a significant difference between the UBP+ exudates and TBP+ exudates with the latter stimulating almost 2 times more J2 hatching (Figure 4.1 B and C). Using PRE from 5-week-old plants, UBP+ exudates treatment reduced hatching by 70% compared to the TBP + exudates treatment. However, when using PRE from 3- and 6-week-old plants, there was no significant differences between the UBP + exudates and the TBP + exudates treatments (Figure 4.1 A and D).

These results suggest that at an *in-vitro* level, the working principle of both the treated and untreated banana paper is similar when the plant is producing root exudates with reduced potency that could reflect less production of HFs as seen when using PRE from

3- and 6-weeks-old plants. However, when the plant produces more HFs i.e., at 4 and 5 weeks, the untreated banana paper is seen to be more effective in reducing hatching of PCN. This could infer that, at high HFs concentration, there is an additional hatch stimulating effect that could be induced by the abamectin in the treated paper. Most studies have described hatch inhibition as one of nematicides mode of action (Deliopoulos *et. al*., 2010; Kearn *et. al*., 2014; Feist *et al.*, 2020). However, other studies have also shown that at low concentrations some nematicides are able to stimulate PCN hatching. The effect of low concentrations of abamectin on PCN hatching is yet to be evaluated. The results could alternatively suggest that saturation of the TBP with abamectin could reduce its efficiency in adsorbing the HF, hence more hatching compared to the UBP treatment.

The effect of the treatments on PCN egg viability was also assessed on the remaining eggs after 8 weeks. No significant difference was observed between the treatments $P > 0.05$. The results indicate that the treatments had no effect on the viability of the eggs suggesting that the low hatching rates linked to the exposure of PRE to banana paper are not caused by an increase of the mortality of the eggs within the cysts.

The difference in hatch stimulation by PRE from plants of different ages is in agreement with other reports that have shown that the physiological status of plants affects the biological activity of root exudates (Rawsthorne and Brodie, 1986; Tefft *et al.*, 1982). The results suggest that exudates from 5-weeks old plants contained the highest number of HFs, while exudates from 6-weeks old plants contained HFs with decreased hatching

activity compared to the others. This could be a result of the plants' flowering onset that leads to a decrease in HFs production in 6-week-old plants or an increase in the production of HIs countering the effect of the HFs. These results are in line with those of Devine and Jones (2003), who also reported increasing hatching activity in exudates from 2- to 5-weeks old, but a decrease in PCN hatching at the onset of potato plant flowering at 6 weeks.

The reduction in PCN hatching in the presence of treated and untreated banana paper compared to the PRE alone suggests an interaction between the banana paper and PRE that significantly reduces the effectivity of HFs present in the exudates. The similarity in hatching of J2s in water alone, TBP + water and UBP + water treatments, suggests that the presence of banana paper does not affect the spontaneous hatch of PCN in water. Therefore, from these results we can infer that when a potato seed is wrapped with treated or untreated banana paper and planted in a PCN infested field, the paper is able to significantly reduce PCN hatching in the first 8 weeks of planting.

Understanding the effect of the banana paper on J2s hatching is essential for PCN control. This is because cysts can stay in the soil, acting as a diapause structure, for more than 20 years. The results indicate that PCN hatching was severely reduced by the banana paper treatments with the treated and untreated banana paper causing 85% and 53% hatch reductions, respectively, in the first 8 weeks of exposure compared to the PRE alone. Since hatching of the encysted J2s is initiated by exposure to HFs present in the PRE, the results suggest that there could be some interaction between the HFs and the untreated

and treated banana paper. This interaction could lead to adsorption of the HFs onto the banana paper or a chemical transformation of the HFs leading to a reduction in the effectivity of the root exudates. In addition, the interaction between the banana paper and PRE alters the composition of the exudates resulting in reduced hatching efficacy, which is reflected in the lack of significant difference between the treatments with water as the test solution (water alone, TBP+ water and UBP+ water). These results rule out any interactions between the banana paper and water as the underlying factor.

Disruption of plant-nematode chemical interaction as a control strategy has been studied by several authors (Chen *et al.*, 1997; González-Pérez *et al.*, 1994; González and Estévez-Braun, 1998; Takasugi *et al.*, 1975). One method of achieving chemical disruption of plant-nematode interaction is the use of compounds that can inhibit nematode hatch i.e hatching inhibitors (HIs). The mode of actions of HIs are not well understood, although they are proposed to either inactivate the HF or competitively bind to the HF receptors in the eggshell. For disruption of *G. rostochiensis* hatching, root diffusates from non-host plant species of the Umbelliferae family (*Anthriseus silvestris, Arehangeliea litoralis, Pimpinella major, Pimpinella saxifrage, Heracleum sibirieum*) have been used (González-Pérez *et al.*, 1994), though the compound(s) responsible for the inhibition was/were not identified. Natural HIs of *G. pallida* were identified as the lignans bursehemin and matairesinol from *Bupleurum salicifolium* (Umbelliferae) (González-Pérez *et al.*, 1994), while the flavonoid, (*E*)-chalcone was later shown to inhibit up to 100% of PCN hatch at concentrations of less than 10 μM (González and Estévez-Braun, 1998). At 50 ppm, asparagusic acid (l,2-dithiolane-4-carboxylic acid)

from *Asparagus* spp. (Liliaceae) inhibited hatch of *G. rostochiensis* and *H. glycines* (Takasugi *et al.*, 1975). The oil of black mustard containing allyl isothiocyanate also irreversibly inhibited the hatch of *G. rostochiensis* at 500 ppm (Ellenby, 1945).

### 4.2 Chemotactic responses of second stage juveniles (J2) to PRE in the presence and absence of banana fiber paper

Potato cyst nematode J2s in the no paper treatment (nylon mesh only) showed significant preference to the stimulus side (PRE) compared to the control side (distilled water). This was seen across the experiments using root exudates from 3-weeks old (A: $X^2$=15.384, df = 5, $P$<0.001), 4-weeks old (B: $X^2$=26.084, df = 2, $P$<0.001, 5-weeks old (C: $X^2$=263.17, df = 11, $P$<0.001, and 6-weeks old (D: $X^2$=94.723, df = 2, $P$<0.001 ) potato plants (Figure 4.2). These results show that the J2s in the no paper treatment are able to fully perceive the chemical attractants present in the PRE (stimulus). This is in agreement with Devine and Jones (2003) hypothesis that some compounds present in the root exudates of potato are attractive to PCN and are used for host location. Other *in-vitro* studies (Devine and Jones, 2001; Farnier *et al.*, 2012) have also demonstrated the chemoattraction of PCN J2s to PRE in bioassays conducted in water, agar and sand, respectively.

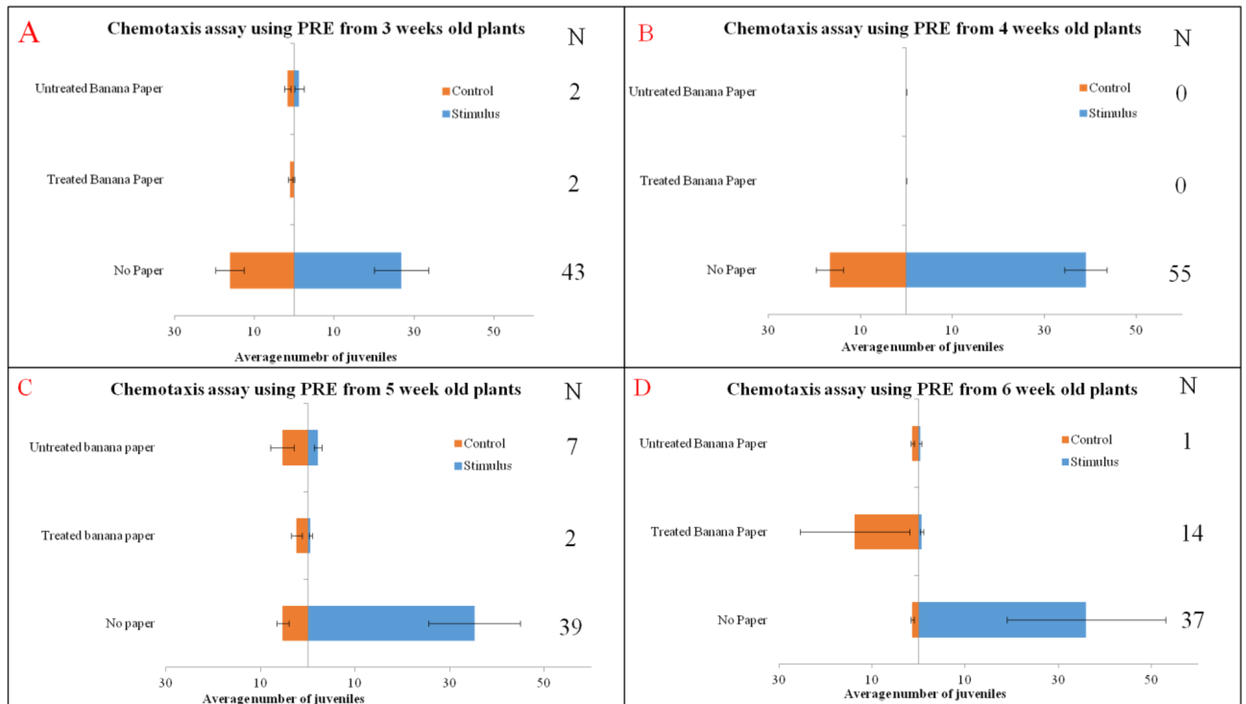



**Figure 4.2:** Chemotaxis response of PCN *G. rostochiensis* infective juveniles (J2s) to PRE (stimulus) in the presence the three treatments: no paper, treated and untreated banana paper compared to control (moist sand). Graph A represent chemotaxis experiment using PRE from 3-week-old plants; B; 4-week-old plants; C; 5-week-old plants and D; 6-week-old plants. N represents the total number of responsive juveniles, i.e., sum of the juveniles from the control and stimulus side.

In the presence of banana paper, the attraction of the J2s to the stimulus (PRE) was greatly reduced as illustrated in Figure 4.2. There were no significant differences in the J2s located in the stimulus and the control side upon application of the treated and untreated paper in all the experiments, i.e. using exudates from 3-6 week-old plants, with an exception of the set up using exudates from 6 week-old plants, where a significant number of nematodes moved to the control side ($X^2$=9.6, d.f=1, $P$=0.001946). This pattern of movement indicates that the J2s moved in a random manner, i.e. not in response to any chemical stimulus, implying that in the presence of banana paper the J2's were unable to perceive the presence of PRE, and locate the chamber with the stimulus.

Comparing the counts of J2s recovered from the stimulus side of the experimental set-up, there were significant differences across the three treatments. The juveniles showed a great preference for the stimulus side in those experimental set-ups where there was no paper treatment compared to the designs where treated and untreated banana papers were used. This was seen in all the four experiments in which exudates from 3-6-week-old plants were tested. Using exudates from 5-week-old plants, the untreated banana paper and treated banana paper significantly reduced ($P < 0.001$) the number of nematodes entering the stimulus side by 94% and 99% respectively, compared to the no paper treatment.

The results suggest that both paper treatments were able to mask the attractant chemical cues that emerge from the stimulus side, preventing J2' perception of its "host", thus disrupting the chemoattraction. Differences were also found between the untreated and treated banana papers, with the latter being 5% more effective in preventing J2s' from entering the stimulus side compared to the former ($P=0.005$). This suggests that in addition to masking the attraction cues from the PRE, the abamectin in the treated banana paper could be further affecting the nematodes by inhibiting their motility. The count of J2 in the control chamber indicates a significant difference between the no paper and the treated paper but not the untreated paper.

These results suggest that the banana paper could be interacting with the PRE resulting in a reduction of chemo-attractants in the exudates. This interaction could be a chemical

process that converts/transforms the chemo-attractants to other compounds that are not recognized by the nematode, or that the banana paper could be adsorbing these compounds and “masking” them by making them unavailable in the soil for the nematodes to detect. Nevertheless, the few numbers of J2s recovered from the control and the stimulus side in the banana paper treatments (represented as N in the graphs on Fig 4.2) could also imply that the paper acts as a physical barrier preventing the chemotactic migration of the juveniles.

Host location is an important phase in the development of sedentary endoparasitic nematodes like PCN. The results imply that wrapping a potato with treated or untreated banana paper enables disruption of the host finding process thus reducing the nematodes' chances of successful parasitism. This is likely to result in less infections and high nematode mortality due to depletion of energy reserves before infection of the host plant. For PCN nematodes, interference with host location process has been achieved by disruption of their chemical perception by blocking their receptors (Bird, 2004; Curtis, 2008). This has been achieved in PCN by Perry *et al.* (2002), who demonstrated that exposure of *G. rostochiensis* to 1% DiTera® prevented chemoreception of potato root diffusates, indicating that DiTera® blocks the amphidal pores or binds to amphidal secretions to interfere with nematode sensory perception. Būda and Čepulytė-Rakauskienė(2015) also demonstrated that pre-exposure of PCN J2s to zinc sulphate solution significantly suppresses their attraction to α-solanine (PCN attractant) suggesting involvement of their chemoreceptors.

### 4.3 Phenologic development of PCN in wrapped and unwrapped potato plants

The count of J2s (Plate 4.1 A) in all the treatments at week 2 (Figure 4.3 A) was very low with the highest observed in the no paper treatment (average of 22 Js) even though there were no significant differences between the treatments. These results suggest that by the second week, majority of the PCN eggs had not hatched, consolidating observation of Devine and Jones (2001) of low potency of root exudates from young plants. At this week, no J3, J4 and adult females were recovered from either of the treatments, as expected. This could be because by the second week, the J2's that had penetrated the roots had not yet established a feeding site to enable them to molt into the consecutive stages. According to Moens *et al.* (2018), molting of J2s to form J3s takes about 7 days after penetration.

B



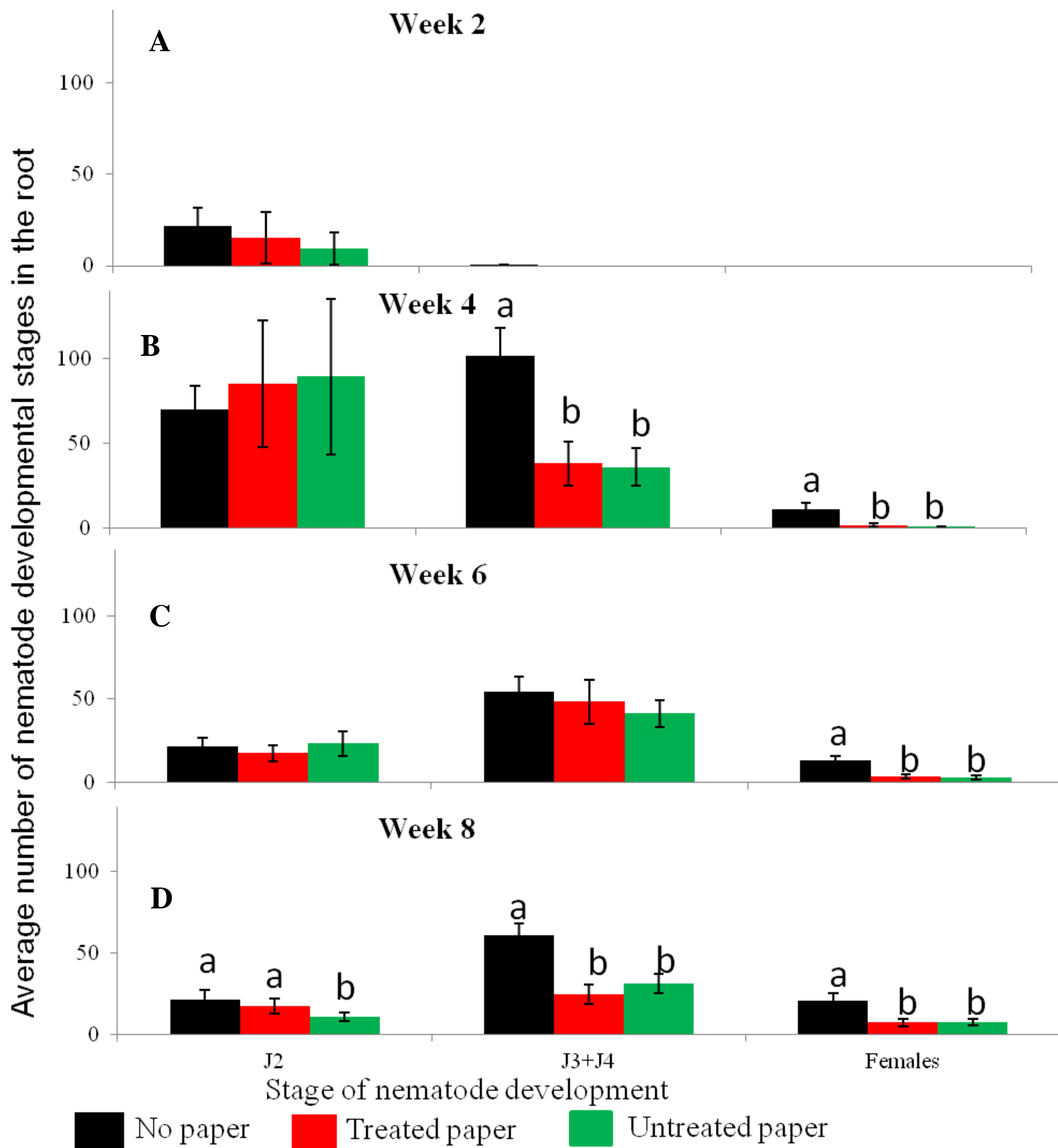



**Figure 4.3:** Different stages of PCN *G. rostochiensis* development on application of different treatments over a duration of 8 weeks. Graphs A, B, C and D represents the available stages of PCN in 2-, 4-, 6- and 8-weeks old potato roots. Different letters refer to significant differences among the three treatments, for each developmental stage and in every week and the same letters shows no significant difference.

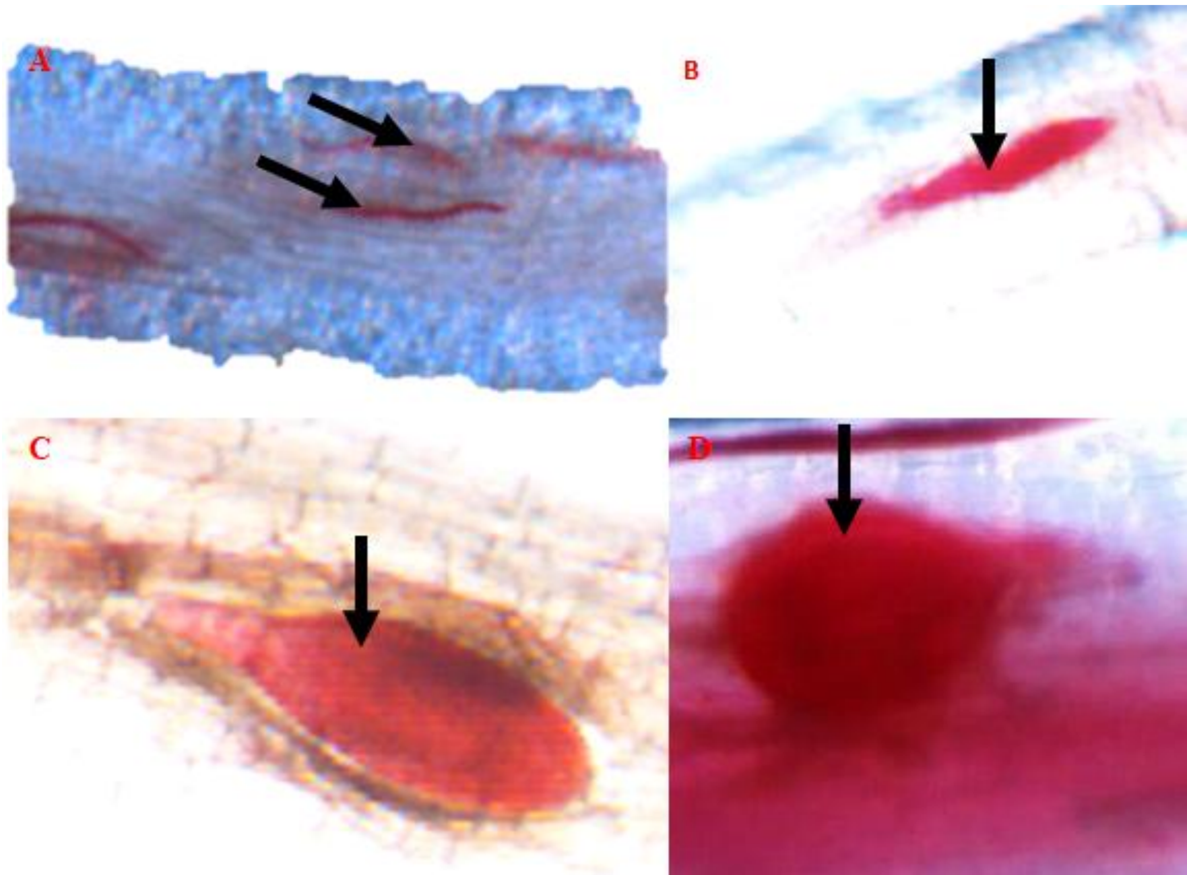



**Plate 4.1:** Different stages of PCN development in potato roots as seen under a dissecting microscope: A) Second stage juveniles; B) Third stage juveniles; C) Young immature females; and D) Mature females.

At week 4 (Figure 4.3 B), the counts of J2s were not significantly different across the three treatments but the no paper treatment had a significantly higher ($P<0.05$) number of J3+J4s (Plate 4.1 B) compared to the treated and untreated paper treatments, which showed lower counts by 25% and 27%, respectively, compared to the no paper treatment. There was also a marginally significant reduction ($P = 0.1$) in females (Plate 4.1 C and D) with the treated and untreated banana paper by 35% and 21% respectively. The formation of the females by the fourth week is earlier than what was reported by (Mimee *et al.*, 2015). This could be related to different environmental conditions and plant varieties

used in this study since previous studies were done with non-African PCN populations and potatoes that take longer to mature, unlike Shangi that is used in the current study. The higher count of J2s and females in the no paper treatments suggests that the plant was more susceptible to infection and had conditions that favored PCN phenological development compared to the treated and untreated banana paper treatments.

At week 6 (Figure 4.3 C), the count of J2s and J3+J4s was not significantly different across the three treatments. This could be due to an increase in the quality or quantity of HFs and chemo-attractants produced by the plant, or due to the onset of the paper degradation that reduces its efficiency, which would make the treated and untreated banana treatments to have comparable effects to no paper treatment. However, the count of females was significantly reduced by the treated paper ($P$=0.006) and untreated paper ($P$=0.004), resulting in a 24% and 22% reduction, respectively, compared to the no paper treatment. Hence, this result suggests that despite infectivity of new J2 and the J3 + J4 population was similar for all the treatments in week 6, the effects of the initial blockage of the J2 infection at the planting stage using paper translated into a reduced number of immature females' half-way down the maturation of the crop (6 weeks).

At week 8 (Figure 4.3 D), significant differences were seen across all the treatments and in all the life stages. There was a marginally significant effect ($P$=0.08) of the untreated banana paper, reducing J2 count by 48% compared to no paper treatment. However, there were no significant differences between the no paper and the treated banana paper suggesting a difference in interactions of the two papers. This result suggests that the

abamectin in the treated paper may be offering additional protection to the plant against PCN infection, as it was observed in the chemotaxis assays. With the J3+J4 counts, the treated and untreated paper showed significant reductions of 37% and 49%, respectively, relative to the no paper control. A similar result was observed at week 8, with the female counts significantly reduced ($P$= 0.0003) by 36% in both the treated and untreated paper treatments.

These results show that when a potato plant is wrapped with treated or untreated banana paper, wrapped tubers are better protected against PCN attacks of hatched infective J2s. This could be an outcome of the interaction between the banana paper and the exudates, leading to delayed hatching and interference in the host location processes as observed in the *in-vitro* bioassays, or as a result of plant priming that could lead to an improvement in the defenses of plants wrapped with the banana papers. The maximum number of J2s in the no paper treatment was observed within the first 4 weeks, compared to the 6$^{th}$ week in the treated and untreated paper treatments. This is consistent with the delay in hatching and infection in the paper treatments.

The time the nematode takes to complete its life cycle is dependent on a number of factors including temperature, humidity, soil moisture, availability of a host among others (Kaczmarek *et al.*, 2014). Most management strategies always aim at disrupting this cycle, especially preventing hatching of J2s and host infection (Ali, 2018; Perry *et al.*, 2000). The current experiment shows that the presence of the paper delays PCN

development and reproduction, and therefore reducing PCN population by preventing next generation formation because less females mature to develop into cysts.

## 4.4 Chemical components present in PRE before and after exposure to banana paper

### 4.4.1 LC-MS/MS analysis of chemical constituents of PRE

Chemical analysis of PRE showed a difference in the number and concentration of the chemical constituents, based on the numbers of peaks and their areas with respect to the age of the plant as shown in appendix (**I**). Potato root exudates from 3 weeks old plants had the fewest number of compounds, while PRE from 5 weeks old plants (Figure 4.4) had the highest number and quantities in the compounds. This difference in root exudates composition could explain the difference in PCN hatching on exposure to the root exudates from plants of different ages as demonstrated in Section 4.1.

The compounds present in PRE were identified on both positive and negative ionization modes. Pure standards were also used to confirm some of the compounds. Four classes of chemical compounds were identified from the root exudates, namely amino acids, phytohormones, triterpenoid and steroidal alkaloids (illustrated in Table 4.1).

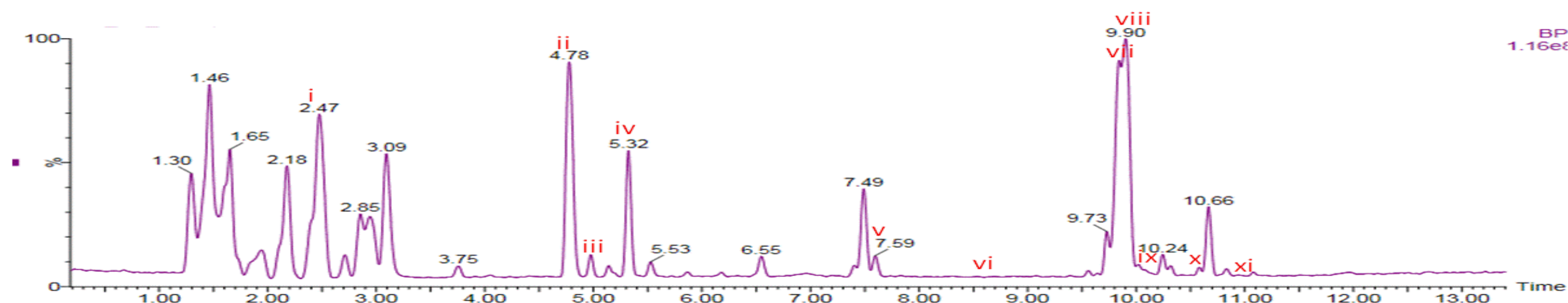


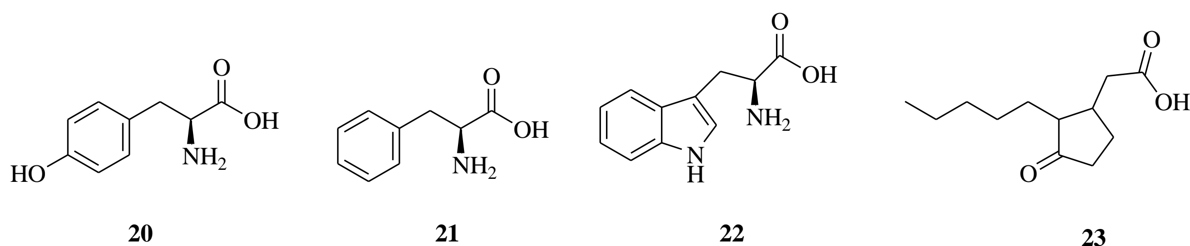

**Figure 4.4:** LC-MS/MS chromatogram of PRE from 5 weeks-old plants with identified compounds

**Table 4.1:** UPLC-MS/MS fragments of identified compounds

| Peak number | Compound | Class of compound | Retention time | Molecular ion (M+H) | Molecular ion (M-H) | Positive mode Fragmentation |
|---|---|---|---|---|---|---|
| i | Tyrosine[b] (**20**) | Amino acid | 2.41 | 182.2 | 180.1 | 119.0, 136.1, 147.1, 165.2 |
| ii | Phenylalanine[b](**21**) | Amino acid | 4.76 | 166.2 | 164.1 | 103.0, 120.1, 149.1, 131.1 |
| iii | Zeatin[b](**10**) | Phytohormone | 5.18 | 220.3 | 218.2 | 202.2, 137.1 |
| iv | Tryptophan[b](**22**) | Amino acid | 5.31 | 205.3 | 203.2 | 188.2, 146.6, 159.3 |
| v | Methyl-dihydrojasmonate[b](**23**) | Phytohormone | 7.57 | 227.3 | 225.2 | 209.2 |
| vi | Solanoeclepin A[b](**2**) | Tetranortriterpene | 8.67 | 499.6 | 497.1 | - |
| vii | α-Solanine[a](**3**) | Steroidal glycoalkaloid | 9.90 | 868.9 | 866.9 | 722.9, 706.8, 560.8, 398.6 |
| viii | α-Chaconine[a](**4**) | Steroidal glycoalkaloid | 9.92 | 852.7 | 850.9 | 706.8, 560.8, 398.6 |
| ix | Solanidine[a](**24**) | Steroidal alkaloid | 10.01 | 398.6 | - | - |
| x | Solasonine[b](**25**) | Steroidal glycoalkaloid | 10.4 | 884.4 | - | 396.3, 576.4, 722.4, 738.4 |
| xi | Solamargine[b](**26**) | Steroidal glycoalkaloid | 11.11 | 868.7 | - | 396.3, 414.3, 560.4, 722.4 |

[a] identified by co-injection with authentic samples

[b] identified by comparison with literature




An aromatic amino acid namely tryrosine (**20**), which eluted at 2.4 min, had a molecular ion peak at m/z 182.2 and 180.1 on both positive $[M+H]^+$ and negative $[M-H]^+$ ionization modes respectively (Appendix II**).** The product ion at m/z 165.2, 147.1, 136.1, 119.0 and 107.0 are attributed to sequential loss of $NH_3$ $[M+H-NH_3]^+$, $H_2O$ $[M+H-NH_3- H_2O]^+$, CO $[M+H- H_2O-CO]^+$ , CO $[M+H-NH_3- H_2O-CO]^+$.and $H_2O$ $[M+H-NH_3- 2H_2O]^+$. Sodium adduct peak was identified at m/z 204.2. The fragments were consistent with those reported in El Aribi *et al.* (2004) and Zhang *et al.* (2019).

Phenylalanine (**21**) eluted at RT 4.7 min with molecular ion at m/z 166.2 and 164.1 for M+H and M-H and product ions at m/z 149.1, 131.1 and 103.0 were formed from sequential loss of $NH_3$ $[M+H-NH_3]^+$, $H_2O$ $[M+H-NH_3- H_2O]^+$ and CO $[M+H-NH_3- H_2O-CO]^+$, respectively (Zhang *et al.*, 2019), and m/z 120.1 which corresponded to loss of water and CO $[M+H-H_2O-CO]^+$ (Appendix III). Sodium and potassium adducts were also identified at m/z 188.2 and 205.2, respectively. Confirmation of the molecular ion peak was confirmed on negative ionization mode (Piraud *et al.*, 2003).

The molecular ion peaks of tryptophan (**22**) was observed at m/z 205.3 in positive $[M+H]^+$ and 203.2 in negative $[M-H]^+$ ionization modes at RT 5.31 min. Mass fragments at m/z 188.2 was due to loss of $NH_3$, and at m/z 146.1 due to loss of $CH_2CO$ resulting

from intermolecular hydroxyl migration from the carboxylic group to the β-carbon (El Aribi *et al.*, 2004; Zhang *et al.*, 2019). The ion at m/z 118.2 is attributed to further fragmentation of m/z 146.2 by loss of CO. Fragmentation ion at m/z 159.3 is attributed to loss of water and carbon monoxide (CO) $[M+H-H_2O-CO]^+$. Sodium adduct peak was also identified at m/z 227.3 (Appendix IV).

Plant hormone zeatin (**10**) identification was based on the molecular ion peaks at m/z $[M+H]^+$ 220.3 on positive and m/z and $[M-H]^+$ 218.2 on negative ionization modes (Appendix V). Fragment ions at m/z 202.2 and 137.1 corresponded to the loss of water $[M+H-H_2O]$ and an adenine derivative ion $C_5H_6N_5^+$. Both sodium and potassium adducts were identified at m/z 242.3 and 258.3, respectively. Similar fragmentation pattern has been reported by (Kirwa *et al.*, 2018). Phytohormone zeatin has also been reported in root exudates of solanaceous crop, tomato, *Solanum lycopersicum* (Kirwa *et al.*, 2018) and *Pinus greggii* (Herrera-Martínez *et al.*, 2014).

Methyl-dihydrojasmonate (**23**) eluted at RT 7.58 min and showed a molecular ion peak at m/z 227.3 in positive and 225.2 in negative ionization modes (Appendix VI). The key fragment at m/z 209.2 corresponded to loss of water $[M+H-H_2O]^+$. Sodium and potassium adducts were also observed at m/z 249.3 and 265.2, respectively.

Identification of the main PCN HF, solanoeclepin A (**2**), was based on the identification of the molecular ion $[M+H]^+$ at m/z 499.6 and formation of the sodium $[M+H+Na]^+$ and $[M+H+K]^+$ potassium adducts at m/z 521.6 and 537.6, respectively (Appendix VII). The

compound eluted at retention time (RT) 6.6 min. The $[M+H]^+$ peak, 499.95 has been reported from *S. tuberosum* cv Desiree root exudates by (Sasaki-Crawley, 2013). On the negative ionization modes, m/z ratio at 497.1 corresponded to the molecular ion peak $[M-H]^+$.

Identification of the steroidal glycoalkaloids, α-solanine (**3**), α-chaconine (**4**), solasonine (**26**) and solamargine (**27**) was based on key fragments obtained from cleavage of the interglycosidic bonds which have low binding energies. The fragments were formed as a result of sugar loss from the protonated compounds as illustrated in Figure 4.5. This form of cleavage was also reported by (Cataldi *et al.*, 2005; Stobiecki *et al.*, 2003) in the fragmentation of α-tomatine, solasodine (**27**) and solanidine **(24**) types of alkaloids.

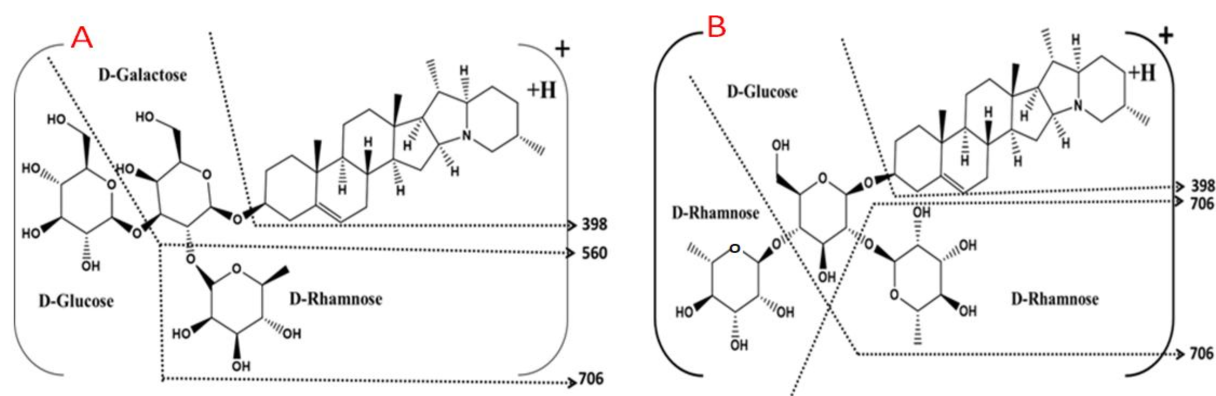


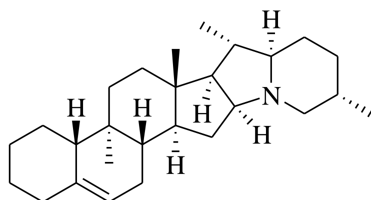

**Figure 4.5:** Structural formulae with the fragmentation patterns of solanine (**A**) and chaconine (**B**)

It is notable that the peaks for the glycoalkaloids, α-chaconine (**4**) and α-solanine (**3**), were not resolved and the two compounds co-eluted at ~ RT 9.9 min. The chemical structures of the two steroidal glycoalkaloids only differ in the sugar moiety attached to

the aglycone, solanidine. In α-solanine, the aglycone is attached to solatriose, while α-chaconine is attached to chacotriose. The similarity between the two compounds also explains the similarity in mass spectra and fragmentation patterns and their poor resolution. α-Solanine (**3**) eluted at RT 9.92 min with a m/z at 866.9 and 868.9 on negative and positive ionization modes respectively. α-Chaconine (**4**) eluted at RT 9.923 min with a m/z at 850.9 and 852.9 on negative and positive ionization modes respectively.

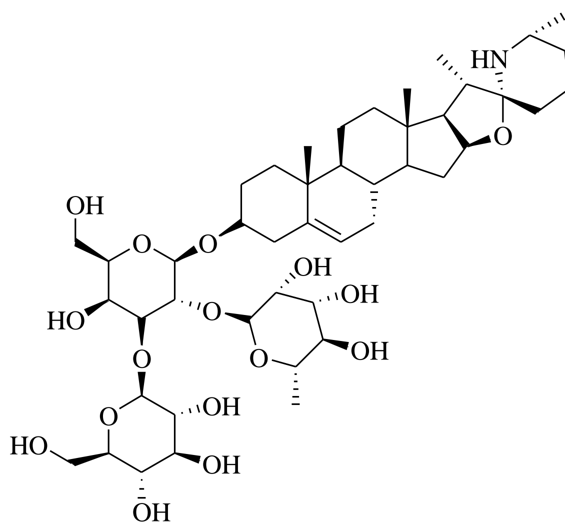
In the mass spectra of α-solanine and α-chaconine (Appendix VIII ), the ions at m/z 722.9, 706.8 and 560.8 are attributed to loss of *L*-rhamnose (Rha) $[M+H\text{-}Rha]^+$, *D*-glucose (Glu) $[M+H\text{-}Glu]^+$, and *L*-rhamnose with *D*-glucose $[M+H\text{-}Rha\text{-}Glu]^+$, respectively (Appendix VIII). The m/z 398.6 ion corresponds to the aglycone $[M+H\text{-}Rha\text{-}Glu\text{-}Gal]^+$ and $[M+H\text{-}Rha\text{-}Rha\text{-}Glu]^+$ ion, formed from losses of all the three monosaccharides in α-solanine and α-chaconine respectively. Identities of the two compounds were confirmed by comparison of mass spectral fragmentation patterns and retention times with authentic standards. Previously, the two compounds were also reported by (Distl and Wink, 2009) when quantifying glycoalkaloids present in tubers of wild *Solanum* species.

Steroidal alkaloid solanidine (**24**) was also identified based on the molecular ion peak $[M+H]^+$ and formation of both sodium and potassium adducts giving m/z 420.1 and 436.6, respectively (Appendix IX). The identity of the compound was further confirmed by co-injection with the standard.

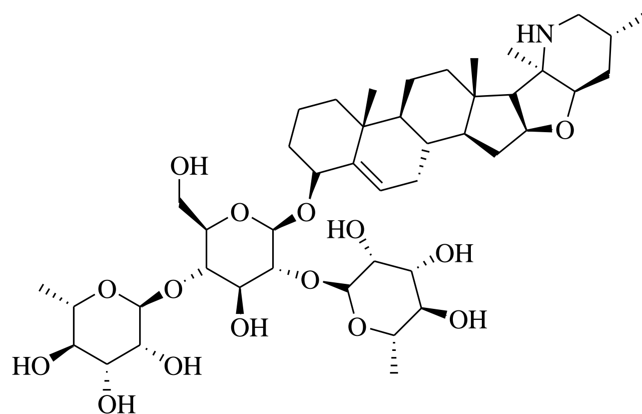


**24**

Identifications of solasonine (**25**) and solamargine (**26**) were based on a similar fragmentation pattern as shown in Figure 4.5. Solasonine (**25**) eluted at RT 10.4 min with a M+H peak at m/z 884.45 and key fragments were formed at m/z 722.4 and 576.3, which correspond to loss of the triose sugars [M+H-Glu] and [M+H-Glu-Rha] respectively (Appendix X).



**25**



**26**

Solamargine (**26**) eluted at RT 11.1min with a M+H peak at m/z 868.5 and key fragments at m/z 396.3, 414.3, 576.3, 704.6, 722.4, and which correspond to loss of the triose sugars and water to give [M+H-Glu-2Rha-$H_2O$], [M+H-2Rha], [M+H-Glu] and [M+H-Rha],

respectively (Appendix XI). The fragments correspond to those identified by Distl and Wink (2009).

Root exudate is an important factor that influences interactions with organisms in the rhizosphere (Canarini *et al.*, 2019; Koo *et al.*, 2005). It consists of organic compounds that include both primary (e.g. sugars, amino acids and organic acids) and secondary metabolites (e.g. alkaloids, flavonoids, terpenes, phenolics) that are passively released into the rhizosphere through the root of living plants across concentration gradients (Grayston *et al.*, 1997; Vranová *et al.*, 2013). Root exudates are utilized by soil dwelling microbes for various biological processes and they can also function as semiochemicals (chemical attractants, repellants, HFs, HIs), growth inhibitors and growth promoters, among other functions (Walker *et al.*, 2003). Chemical analysis of potato root exudate identified different classes of compounds, including amino acids, phytohormones, steroidal glycoalkaloids and steroidal alkaloids, similar to previous findings (Kirwa *et al.*, 2018; Koo *et al.*, 2005; Li *et al.*, 2013).

#### 4.4.2. Effect of selected authentic compounds from potato root exudate on PCN hatching

The effect of the compounds identified from the PRE on PCN hatching was determined. PCN J2 hatching response was highest with the steroidal glycoalkaloids (SGAs), intermediate with the steroidal alkaloids (aglycones), and lowest with the amino acids and phytohormones as shown in Figure 4.6 and Appendix XII. For the SGAs, the PCN was more sensitive to lower concentrations of α-chaconine (**4**), i.e. at 0.2 µg/ml ($\chi^2$=

10.39, df = 1, $P < 0.05$) and at 0.4 µg/ml ($\chi^2 = 90.12$, df = 1, $P < 0.001$) than α-solanine (**3**).

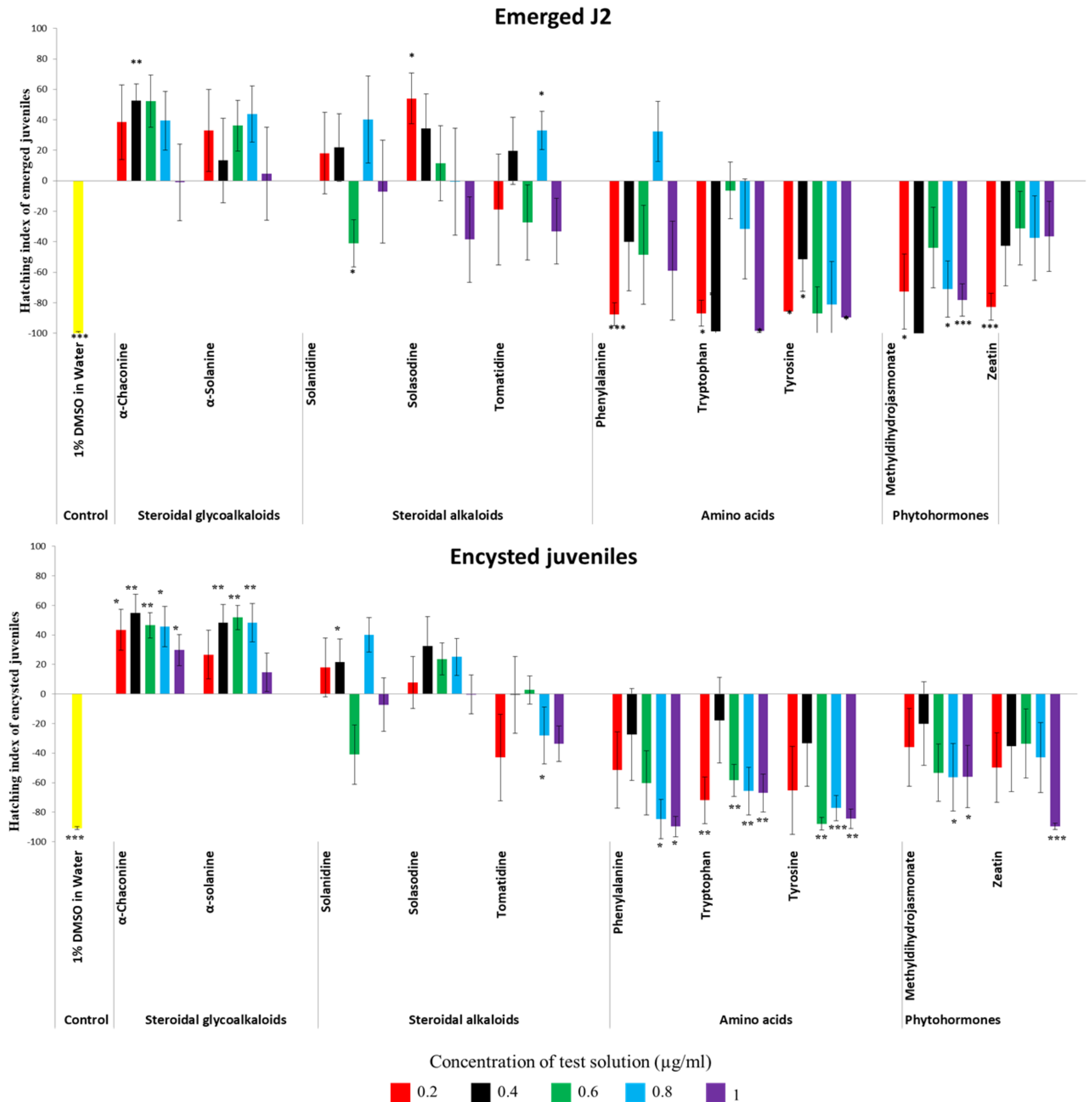



**Figure 4.6:** Hatching indices: A) Emerged J2s, and B) Encysted J2s of PCN, *G. rostochiensis*, in response to ten compounds at five concentrations (µg/ml) compared to the positive control (PRE).

In the present study, α-solanine (**3**) and α-chaconine (**4**) elicited the highest PCN hatching response, consistent with the previous findings (Byrne et al., 1998; Devine and Jones, 2000; Devine et al., 1996). Structurally, α-solanine and α-chaconine are similar. They bear the same aglycone, i.e. solanidine, which is attached to three different sugar moieties. For example, the triose of α-solanine consists of two six-carbon sugars, *D*-glucose and *D*-galactose and one five-carbon sugar *L*-rhamnose. On the other hand, the triose of α-chaconine consists of one six-carbon sugar *D*-glucose, and two five-carbon sugar *L*-rhamnose (Ghisalberti, 2006).

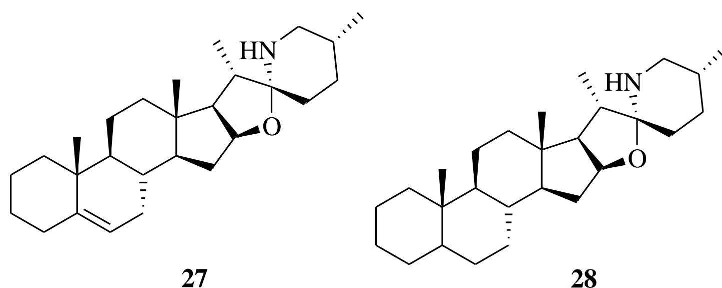
The fact that the two SGAs elicited similar hatching response patterns at different concentrations, suggests that the sugar type and its stereo-structure linked to the aglycone may influence J2 stimulation response. Although the two SGAs are highly oxygenated and polar, the sugar types and their configuration may determine their binding patterns to receptors via hydrogen bonding to stimulate J2 hatch (Torto *et al.*, 2018a). It appears that α-chaconine has the correct configuration to bind to the receptors to stimulate J2 hatch at lower concentrations, whereas higher concentrations of α-solanine are needed to bind to the hatching receptors. It is interesting that the majority of J2 that hatched in response to the SGAs remained encysted. This suggests that additional chemical cues are needed to stimulate optimal emergence, or host location of J2s once they have hatched. Previously, SGAs have been associated with growth inhibition of various bacterial (Seipke and Loria, 2008) and fungal species (Munafo and Gianfagna, 2011). The fact that we found SGAs in the PRE of plants grown in sterilized autoclaved sand, suggests that the plant may be

priming itself against microbial attack. As such, it appears that the PCN has, over time, evolved to ‘eavesdrop’ into the plant defense priming system to hatch in response to varying concentrations of SGAs, as demonstrated in the present study.

PCN hatching indices of the aglycones varied with the concentration of the compound. Solasodine (**27**) elicited a higher emergence of J2 across the different concentrations than solanidine, e.g. at 0.2 µg/ml ($\chi^2$= 100.25, df = 1, $P < 0.001$), at 0.4 µg/ml; ($\chi^2$= 32.70, df = 1, $P< 0.001$), compared with tomatidine at 0.2 µg/ml ($\chi^2$= 37.92, df = 1, $P < 0.001$), and at 0.6 µg/ml ($\chi^2$= 4.96, df = 1, $P < 0.05$) eliciting the lowest response (Figure 4.7 A). However, PCN response to the aglycones was dominated by encysted rather than emerged J2, comparable to responses with solasodine and solanidine (Figure 4.7 B; Appendix XII).

Although PCN hatched in response to the steroidal alkaloids (aglycones), it was lower than with the SGAs. This is likely because they are less oxygenated and therefore less polar than the SGAs. As with the SGAs, most of the hatched J2 remained in the cyst, confirming that additional cues are required to stimulate optimal emergence. However, of the aglycones, tomatidine (**28**) elicited the lowest hatching response. Structurally, tomatidine is less polar than solasodine and solanidine because the latter two bear an olefinic bond in ring B of the molecule. Consequently, future studies need undertake structure-activity relationships, such as the influence of oxygenation and presence of olefinic bonds in triterpenoids identified in the potato root exudate and the exudates of other host plants in PCN hatching response.

A recent study identified solasodine and tomatidine in the root exudate of tomato, an alternative host for PCN, which induced significant stylet thrusting in the plant parasitic nematode, *M. incognita,* even though they did not elicit attraction (Kirwa et al., 2018). In another study, pre-exposure of *M. incognita* to the quinolone alkaloids waltherione A and waltherione E, isolated from the aerial part of *Triumfetta grandidens*, significantly inhibited egg hatch (Jang et al., 2015). These findings demonstrate the parsimonious role of specific host root exudate compounds on the behavior of different plant parasitic nematodes-specific SGAs and their aglycones as HFs in *G. rostochiensis*, but egg HIs and J2 stylet thrusting stimulants in *M. incognita*. Hence, it would be interesting to further examine these compounds at the same concentrations, either individually or in blends, to elucidate their full role in the behavior of different species of PPNs.




Of the amino acids, phenylalanine stimulated a higher J2 hatch compared to tryptophan and tyrosine but only for the emerged J2 (Figure 4.6 A, Appendix XII), whereas there was no detectable stimulation of encysted J2 hatch for all the amino acids. A similar pattern was observed for the phytohormones (Figure 4.6 A, Appendix XII).

Of the four classes of compounds detected in PRE, the amino acids and phytohormones had the least effect on egg hatch. Although these compounds are also polar in nature, they are associated with the root exudates of several plants, including tomato (Kirwa et al., 2018), maize (Carvalhais et al., 2011), sorghum and cowpea (Odunfa, 1979), cotton (Sulochana, 1962), rice (Bacilio-Jiménez et al., 2003), and peanut (Li et al., 2013), among others. As such, they may be perceived by the PCN as non-specific chemo-stimulants. However, it is possible that they may contribute as important background signals to the SGAs and their aglycones in the root exudates to stimulate PCN hatching, which needs to be investigated. Further testing of the naturally occurring concentrations of the SGAs, aglycones, amino acids, phytohormones and unidentified compounds may shed more light on the role of the potato root exudate and its metabolites in PCN hatching.

### 4.4.3 Chemical composition of PRE after exposure to banana paper

When the PRE was exposed to treated and untreated banana paper, their chemical profile changed. This was evident by the reduction in peak areas and absence of some peaks especially the more polar compounds eluting in the first 12 min. as illustrated in figure 4.7.

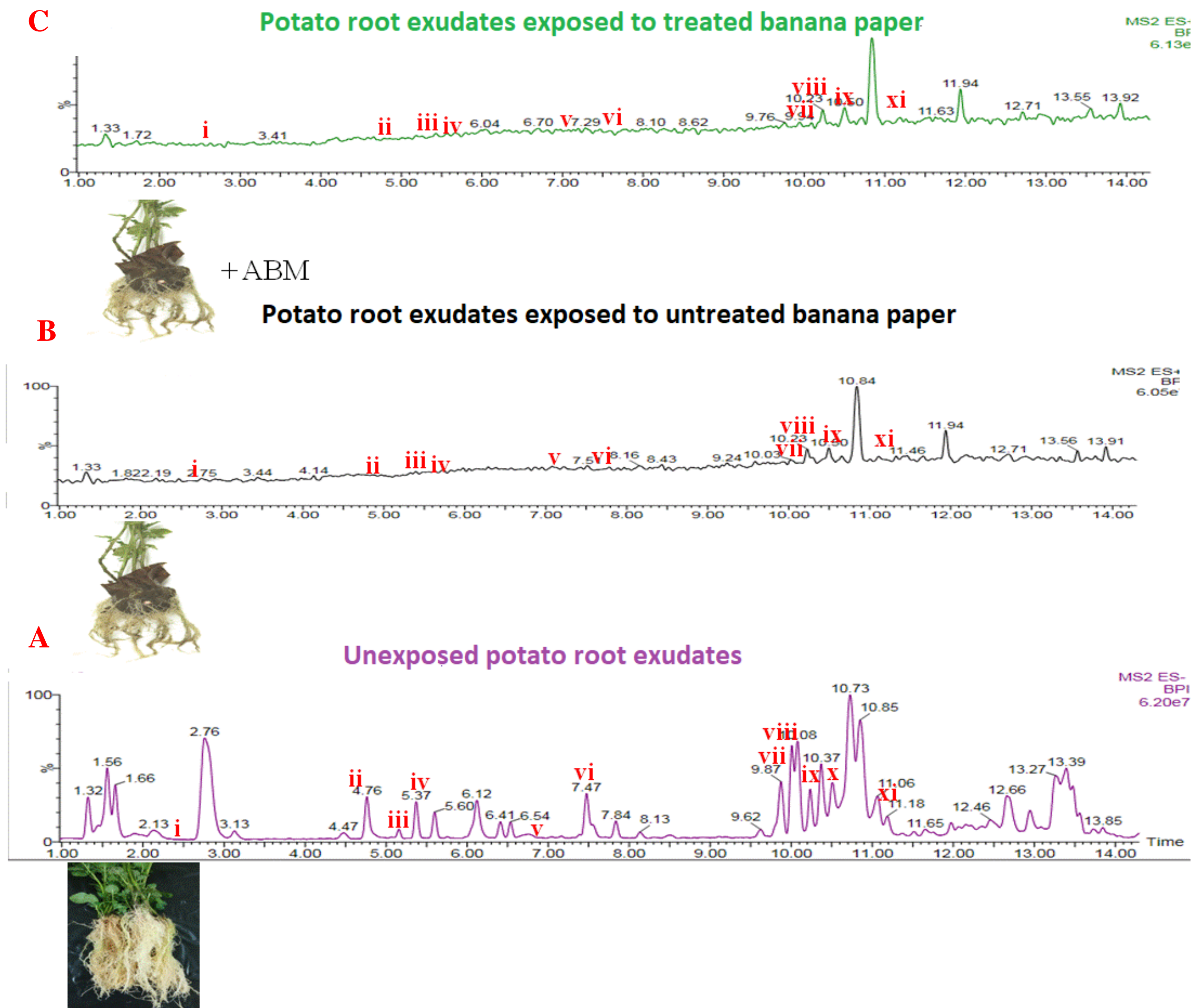



**Figure 4.7:** LC-QqQ-MS chromatograms comparing the composition of PRE before (A) and after exposure to the untreated banana paper (B) and treated banana paper (C). Roman numbers i-xi represents compounds identified in table 4.1

The results from this experiment suggest that an interaction between the PRE and the banana paper may have led to adsorption of the polar compounds in the exudates on the paper.

### 4.4.4 Proof of adsorption of potato root exudate components by banana fiber

Chemical analysis of the PRE exposed banana fiber paper extract showed the presence of PRE constituents including the earlier identified amino acids, phytohormones, tetranortriterpenoid, steroidal glycoalkaloids and steroidal alkaloid (Figure 4.8). The ability to recover the compounds from the paper suggests that the interaction between the banana fiber paper and PRE was physical and did not involve a change in the nature of the compounds. Additionally, from the compounds detected on the banana paper exposed to PRE extract, there was no evidence that suggested an alteration in the structures of any of the compounds present in the PRE.

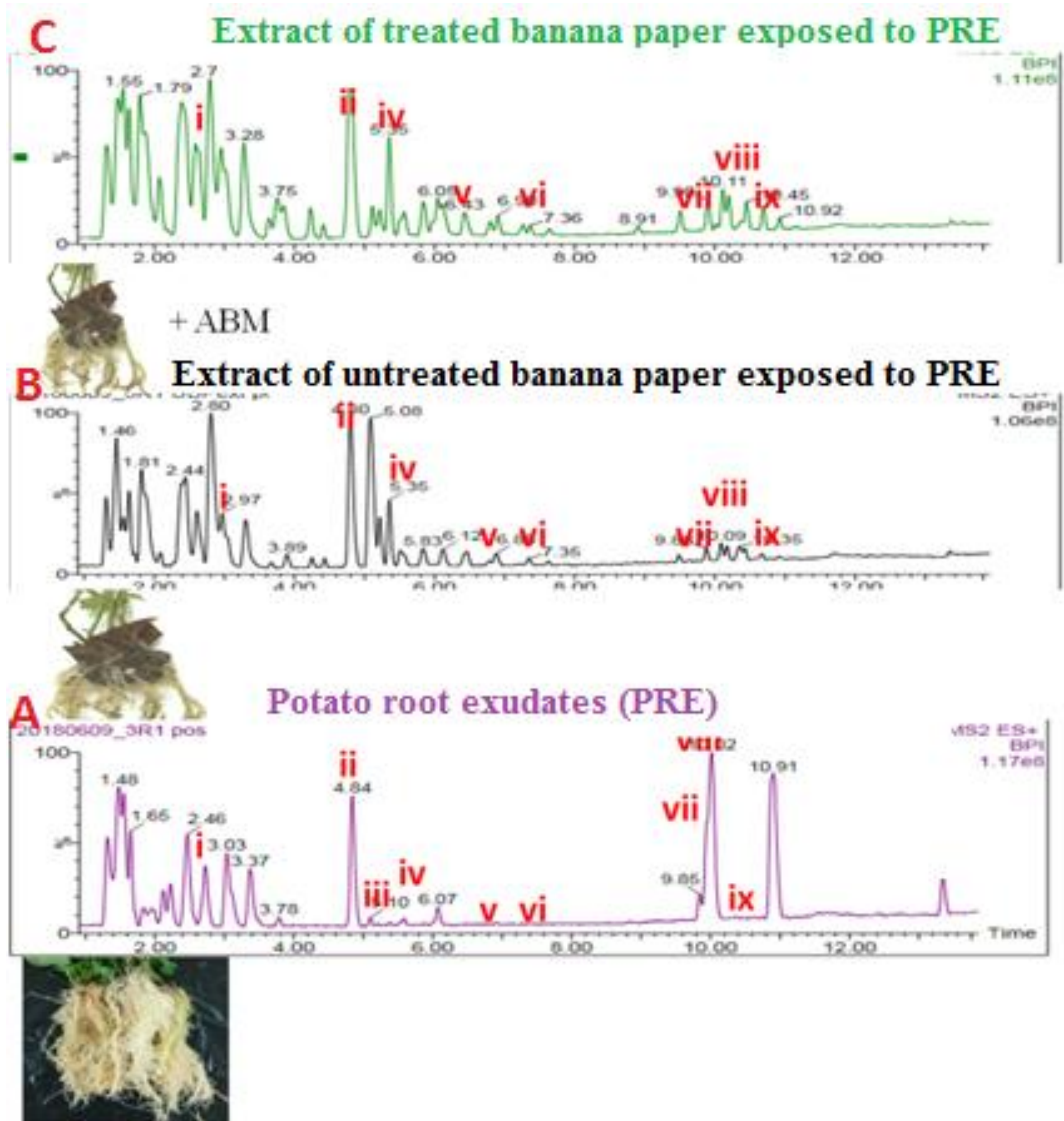



**Figure 4.8:** LC-QqQ-MS chromatograms comparing components of PRE (A) and extracts of untreated (B) and treated (C) banana paper exposed to PRE. Roman numbers i-xi represents compounds identified in table 4.1

To determine if the recovered compounds still had some biological activity, a hatching assay was carried out with the paper extracts. The hatching assay showed a significant difference $p \leq 0.05$ in hatch stimulation from PRE exposed banana paper compared to a

control (water) (Figure 4.9). The results support the LC-MS/MS observations, which showed that the interaction of the paper and PRE does not lead to the transformation of the compounds.

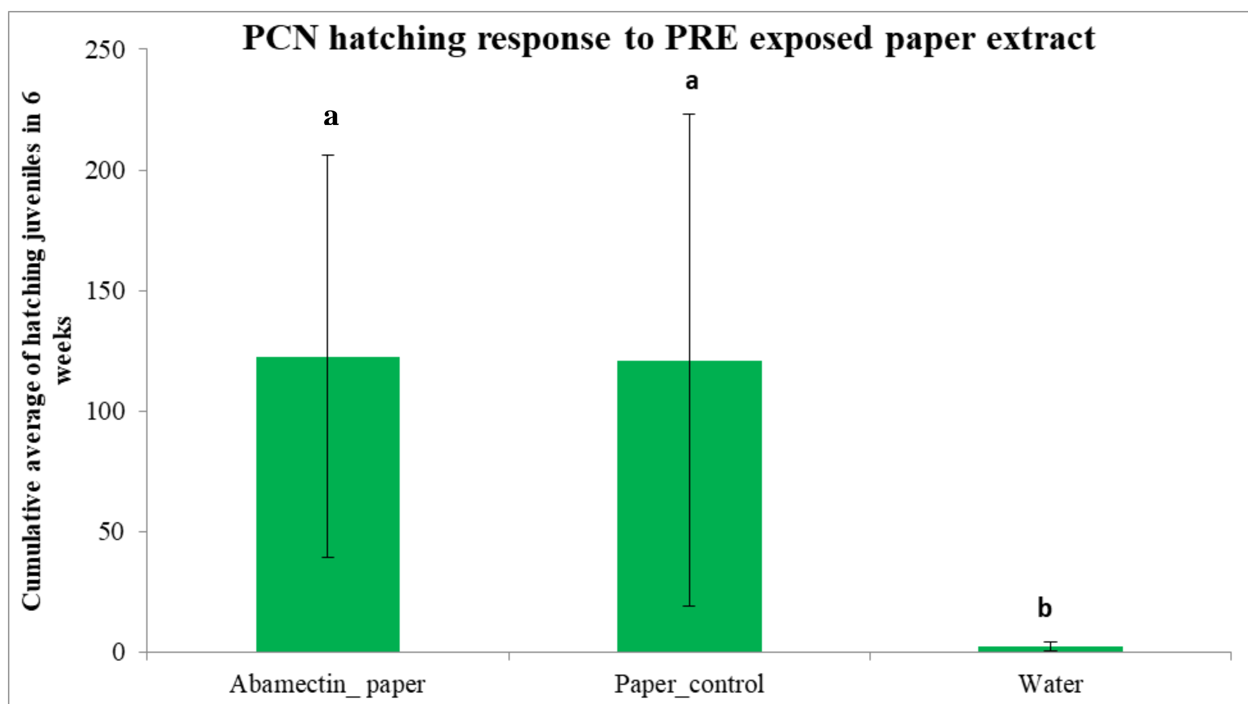



**Figure 4.9:** Hatching response of PCN *G. rostochiensis* to extract of treated and untreated banana paper after exposure to PRE.

### 4.4.5 Chemical composition of PRE before and after exposure to pure cellulose

Chemical analyses of PRE before and after exposure to pure cellulose revealed that most of the components of PRE, including PCN HFs α-solanine (**3**), α-chaconine (**4**) and solanidine (**24**), were adsorbed onto the cellulose (Figure 4.10). This confirms our hypothesis that the cellulose present in the banana fiber paper is responsible for the adsorption.

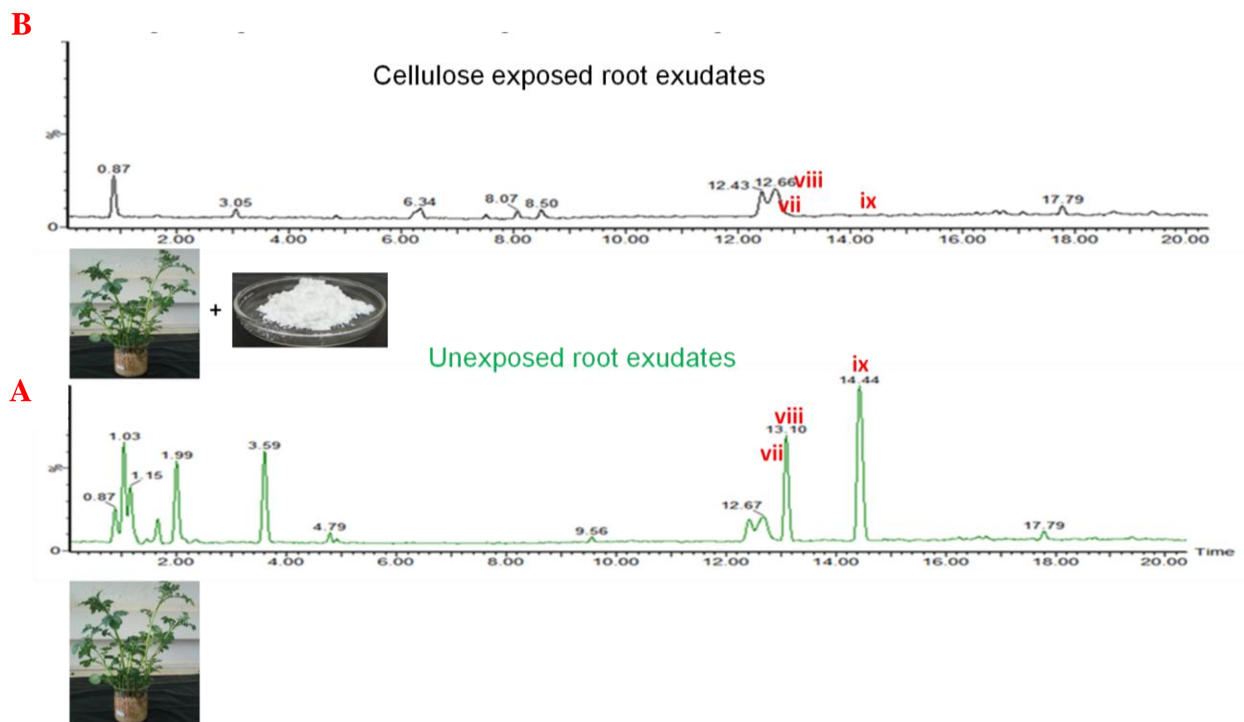



**Figure 4.10:** LC-QqQ-MS chromatograms of PRE before (A) and after (B) exposure to pure cellulose.

Cellulose contains a number of hydroxyl moieties and oxygen atoms, that form intermolecular and intramolecular hydrogen bonds between the same and neighboring cellulose chains and provides stiffness to cellulose chains (Kocherbitov *et al.*, 2008). Since most of the components of PRE are polar, hydrogen bonding to the cellulose matrix in the paper could potentially explain why the compounds could not be detected after exposure to the banana fiber paper. Figure 4.11 illustrates the potential reaction centers in solanoeclepin A and pure cellulose that could be interacting by hydrogen bonding.

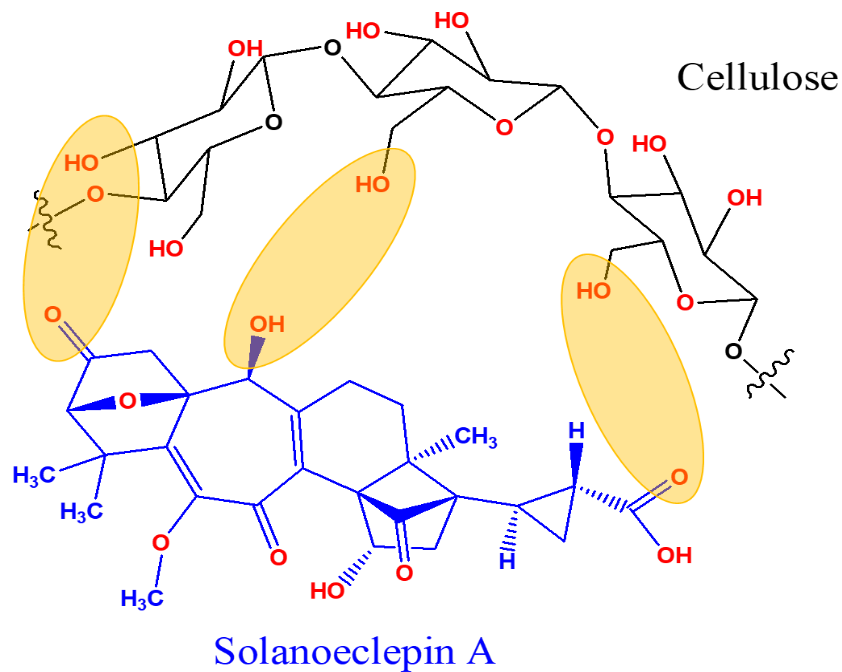



**Figure 4.11:** Potential intermolecular hydrogen bonding sites between cellulose and polar component of PRE (Solanoeclepin A). The elements in red highlight all possible interaction sites by hydrogen bonding between the two molecules.

Cellulose contains a number of adsorption sites and has been used in the removal of various substances including organic materials, water, and metal ions (Abdolali *et al.*, 2014; Gupta *et al.*, 2016). László *et al.* (1999) studied the removal of phenol from aqueous solution using a porous carbon prepared from packing material as a source of cellulose. The study suggested that this adsorption activity depended on the surface functional groups too, especially on oxygen containing moieties. A clearer understanding of the interactions of the banana fiber paper and PRE can be achieved through studies focused on the structure of the paper.

# CHAPTER FIVE

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 CONCLUSIONS

In summary, our study suggests that banana fiber paper disrupts potato plant – PCN *G. rostochiensis* chemical interaction. This is evident from the hatching assays that suggested a reduction in PRE effectiveness after exposure to banana fiber paper leading to a reduction in PCN hatching. The chemotaxis experiment also suggested that the banana paper masks the chemical cues responsible for PCN attraction leading to a disruption of the host finding phase of the nematode. The chemotaxis assays also showed that the treated banana paper was more efficient in reducing the number of J2s in the control and the stimulus side pointing towards and additional effect of abamectin in interfering with J2 motility. Finally, the development experiment also indicates that the banana fiber paper can delay PCN progression development resulting in delayed formation of females.

Chemical analyses of PRE before and after exposure to banana paper indicate that the paper adsorbs the polar components in the PRE inclusive of PCN HFs (solanoeclepin A, α-solanine and α-chaconine). The ability to recover bioactive root exudate compounds from banana paper exposed to PRE also shows the interaction of banana paper and PRE does not structurally modify PRE components. Microcrystalline cellulose was also found to have a similar adsorption effect as the banana paper suggesting that the cellulose component of banana fiber paper is responsible for the adsorption.

Therefore, the Wrap & Plant technology could be an effective and environmentally friendly method for small-holder farmers in Kenya, for increasing potato yields while reducing the damaging effect of PCN. While it would be advisable for farmers not to grow potatoes in case of PCN infestation, the W&P technology could be used together with other PCN control mechanism (i.e., resistant potato cultivars) in rotation programs to manage PCN and bring the inoculum levels below the economic damage threshold. This would be particularly beneficial in Kenya, where farmers have shown reluctance to apply long crop rotation programs. The current study also identifies SGAs and their aglycones as potential candidates for induction of PCN 'suicidal hatch'. This opens up avenues for exploitation of crops such as night shades which produce these compounds during rotations to reduce nematode densities in the soil.

## 5.2 RECOMMENDATIONS

i. The banana fiber paper application in management of soil-borne pests should be widened to other PPNs.

### 5.2.2 Areas for further research

i. Both volatile and non-volatile compounds exuded from the host plant roots have been shown to influence PCN chemotaxis. Further studies should investigate the interaction of banana fiber paper with the volatile organic compounds exuded from potato roots.

ii. Banana fiber paper is majorly made up of cellulose which makes it biodegradable and hence environmentally benign. However, understanding its effect on the soil microbiota would also expound our knowledge on its mechanism of action.

iii. The current study has mainly focused on effect of the banana fiber paper on PCN chemical interactions. Further studies should investigate the effect of the banana paper on the genetic make-up of the plant.

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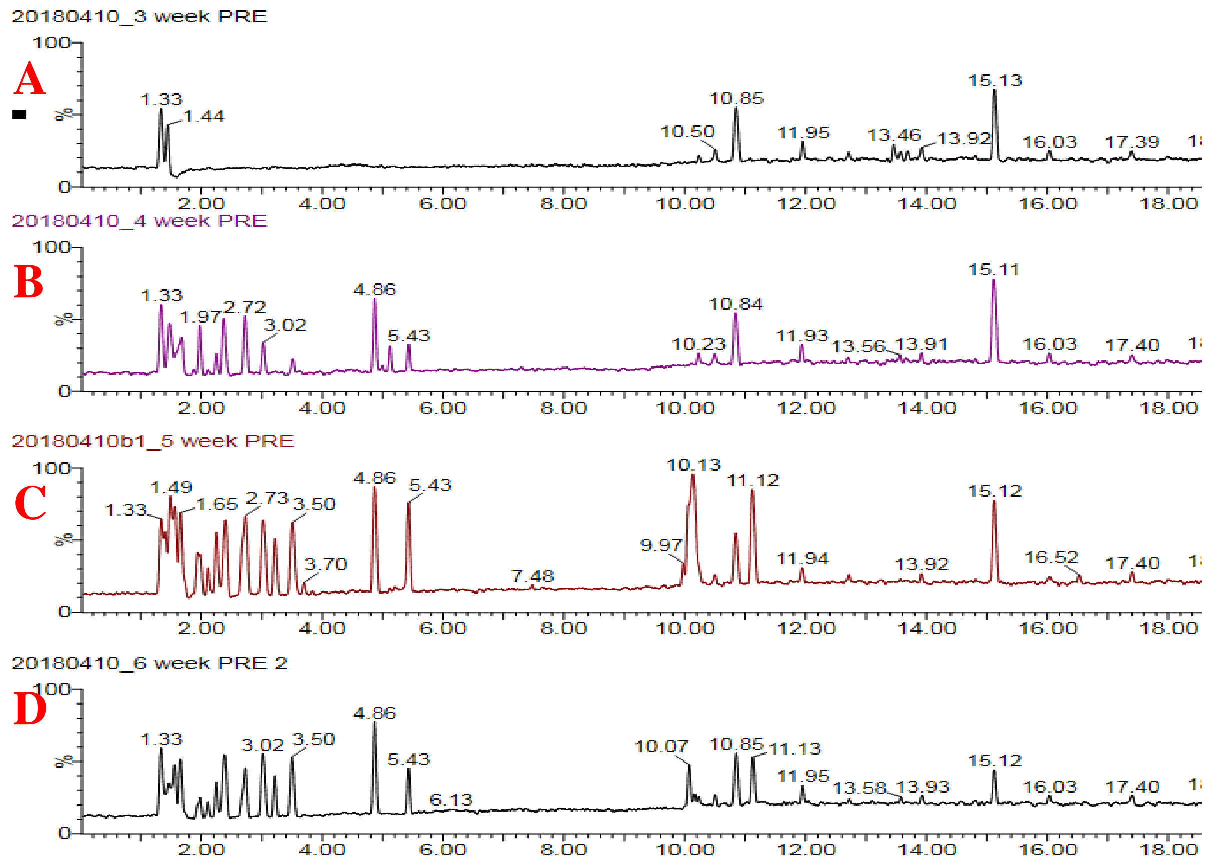
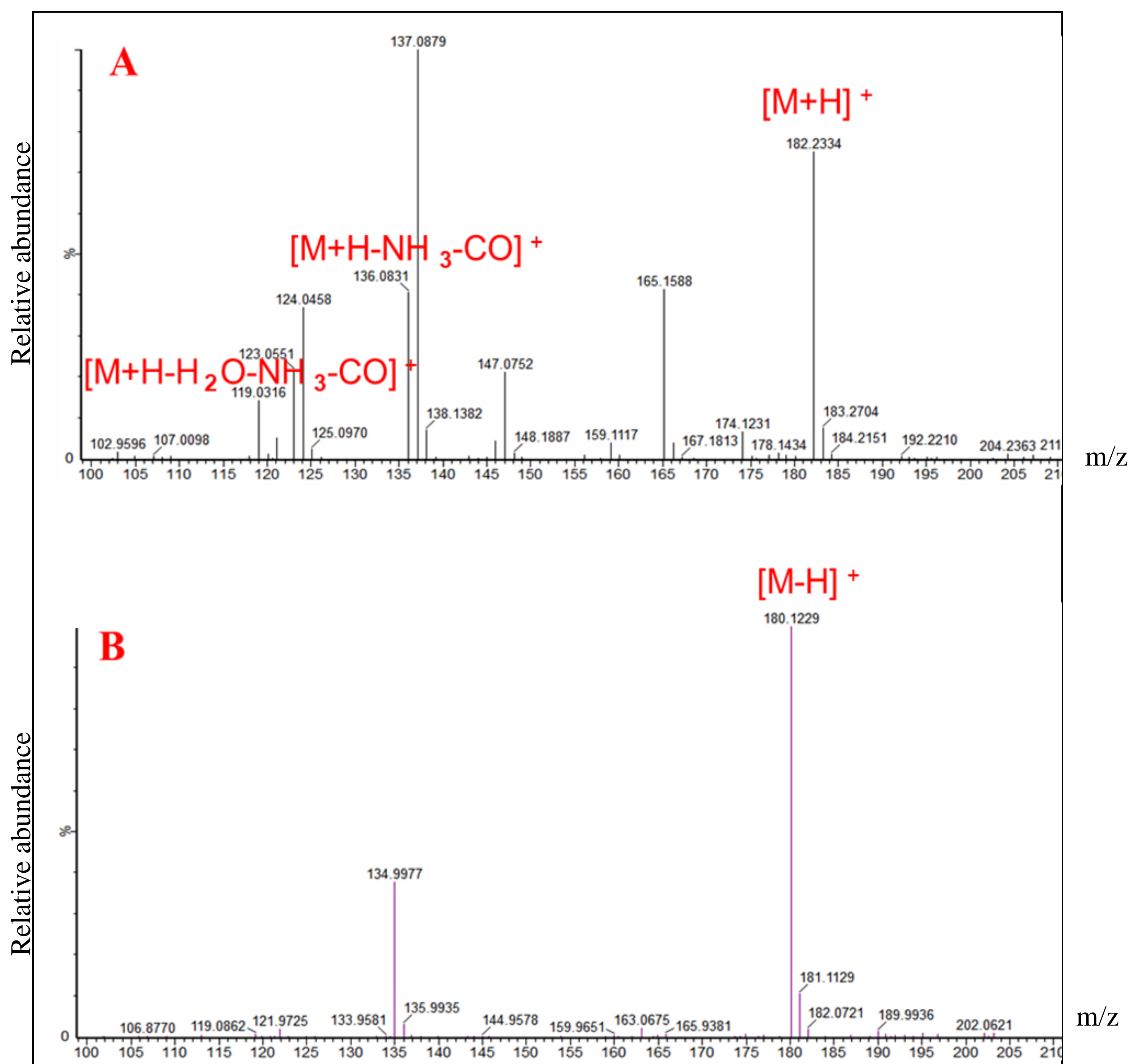
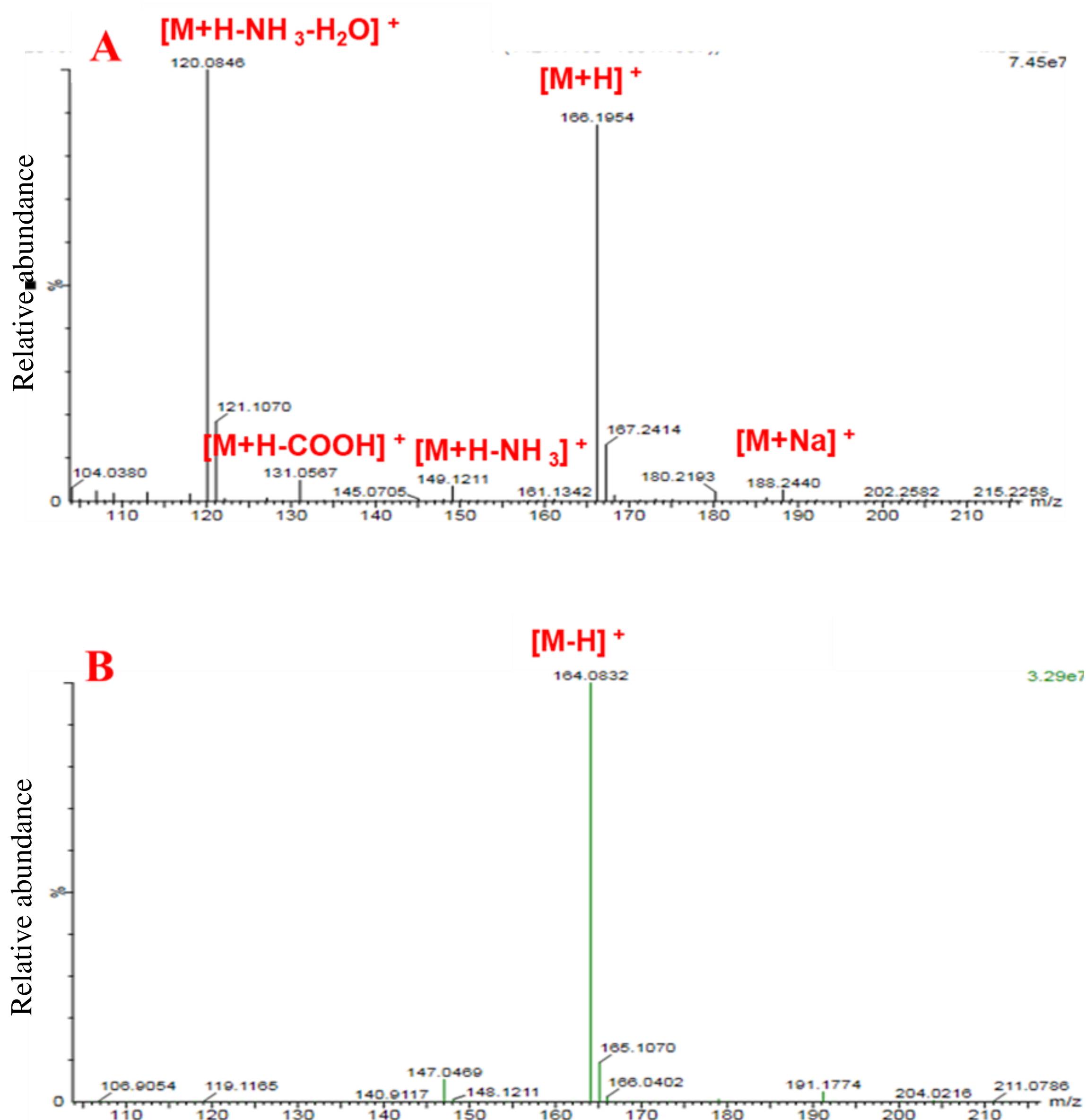
# APPENDICES



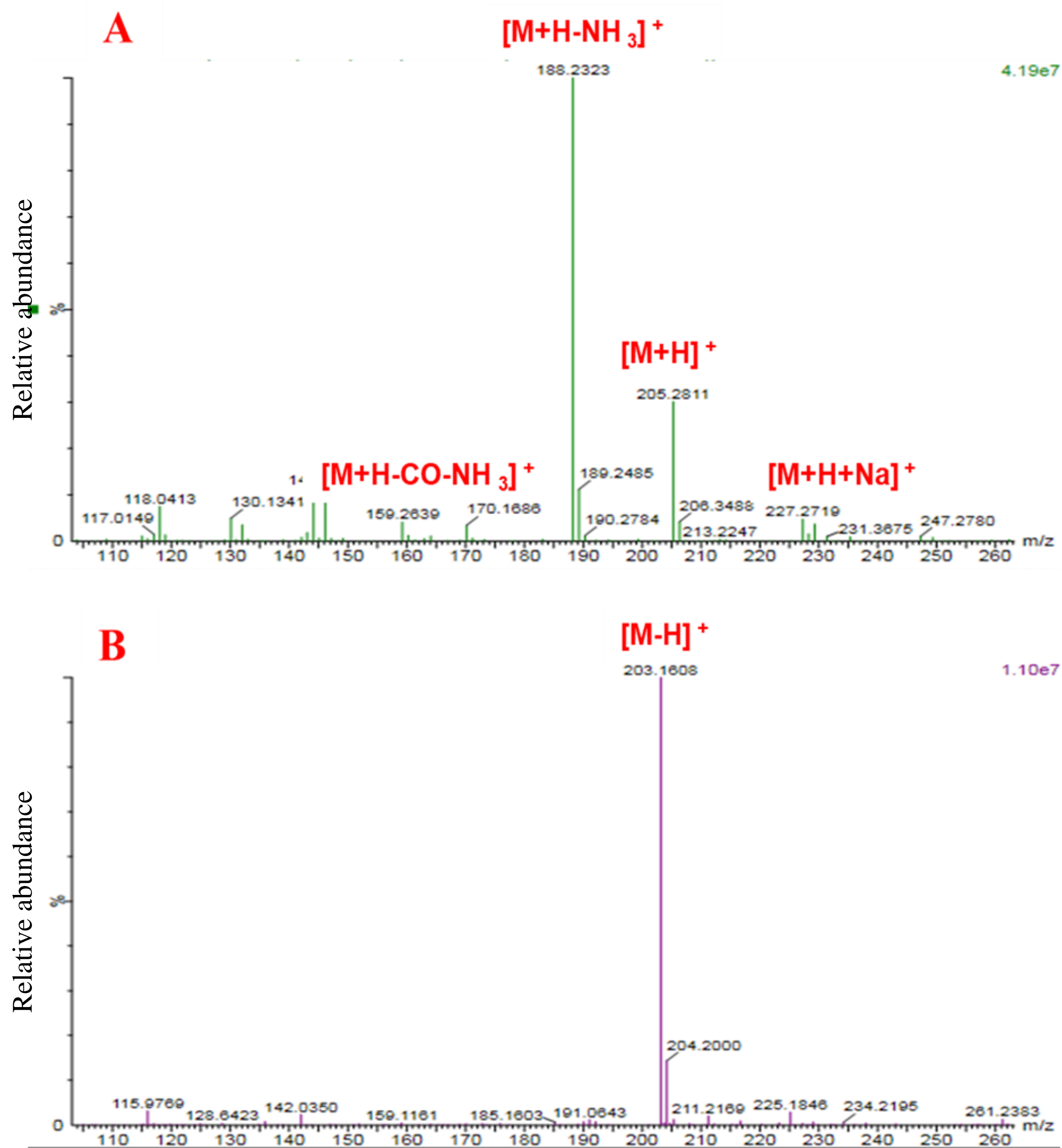

**Appendix I:** LC-MS/MS chromatographs of PRE from A) 3 weeks old plants, B) 4 weeks old plants, C) 5 weeks old plants and D) 6 weeks old plants



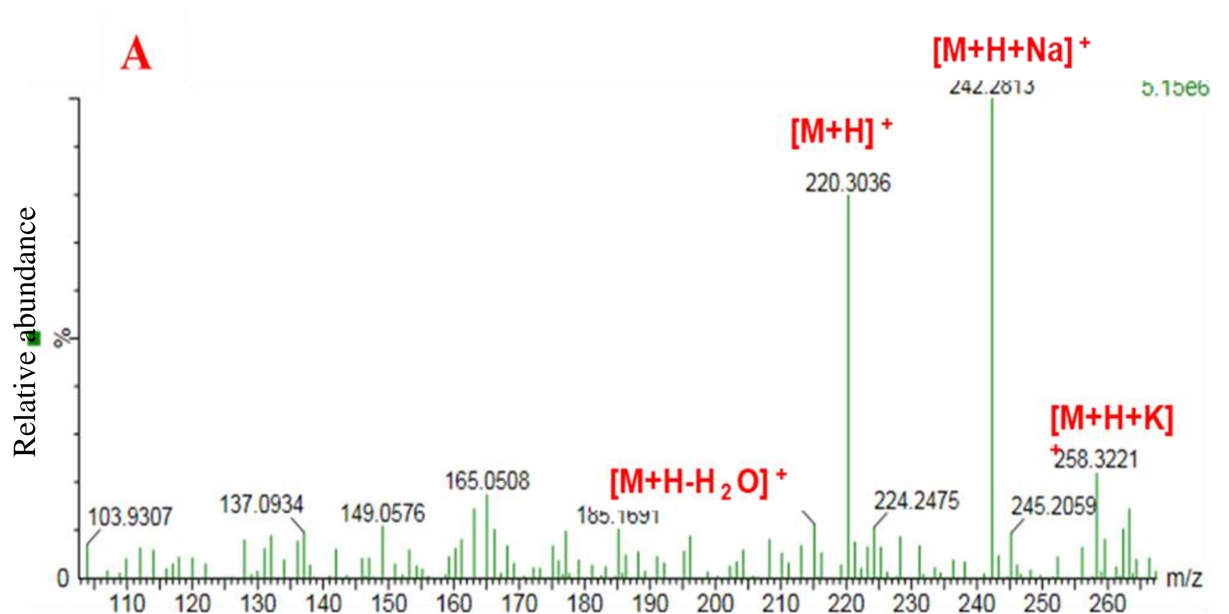

**Appendix II:** Mass spectrum showing the ESI fragmentation pattern of tyrosine positive (A) and negative (B) ionization modes

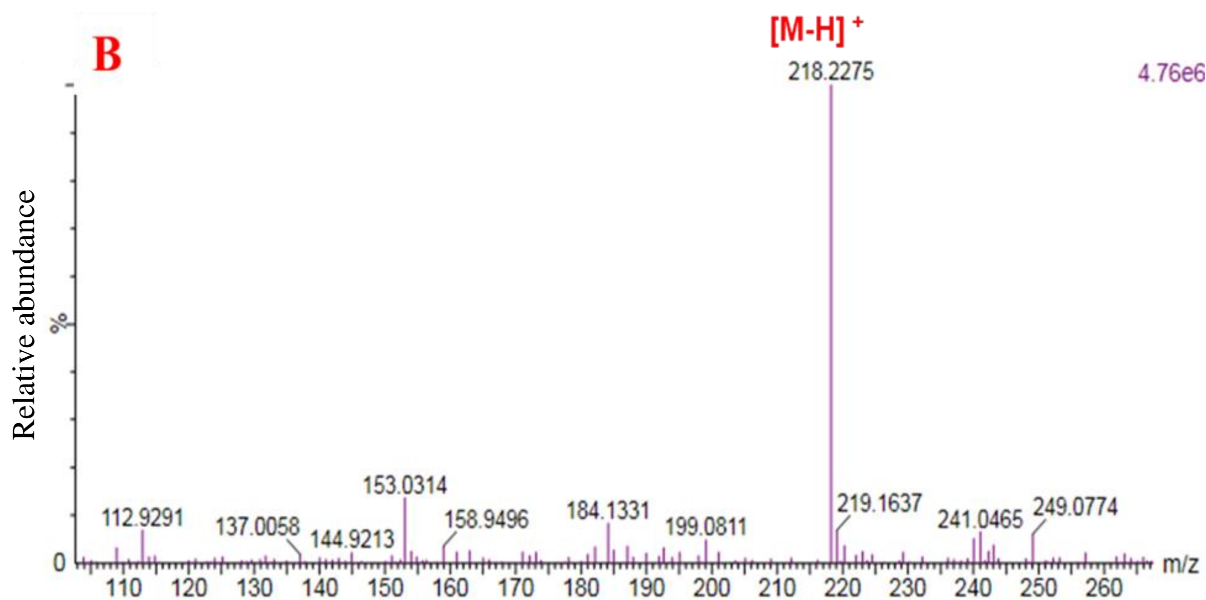



**Appendix III:** Mass spectrum showing the ESI fragmentation pattern of phenylalanine positive (A) and negative (B) ionization modes

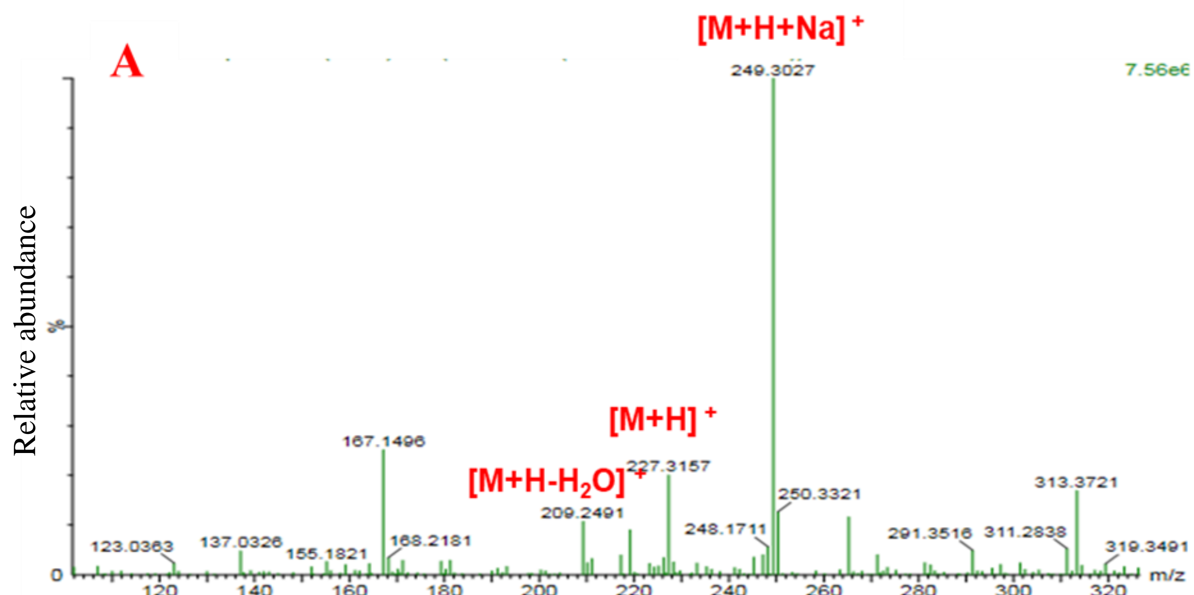


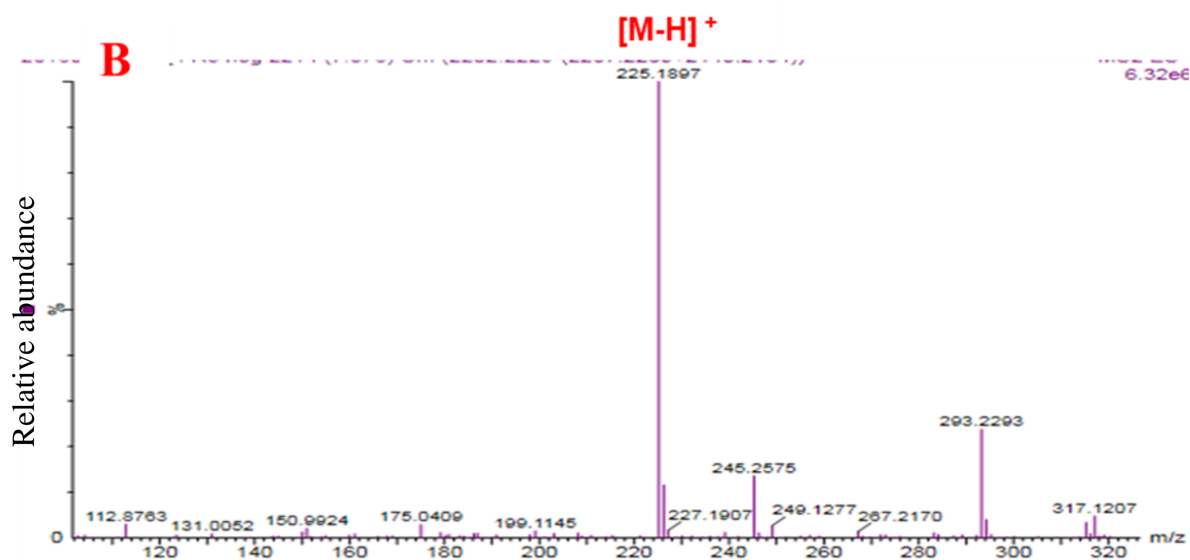

**Appendix IV:** Mass spectrum showing the ESI fragmentation pattern of tryptophan positive (A) and negative (B) ionization modes



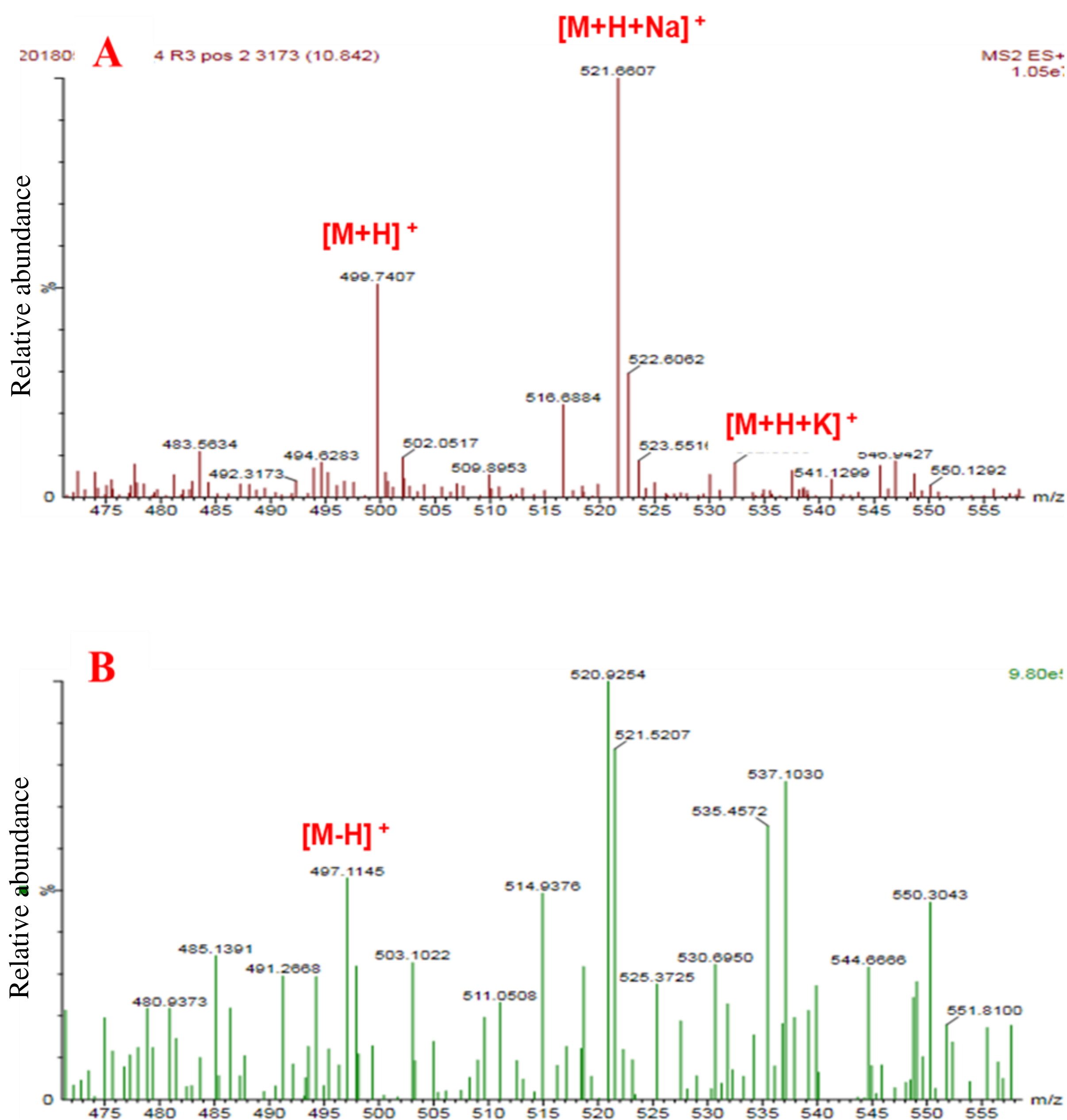




**Appendix V:** Mass spectrum showing the ESI fragmentation pattern of zeatin positive (A) and negative (B) ionization modes

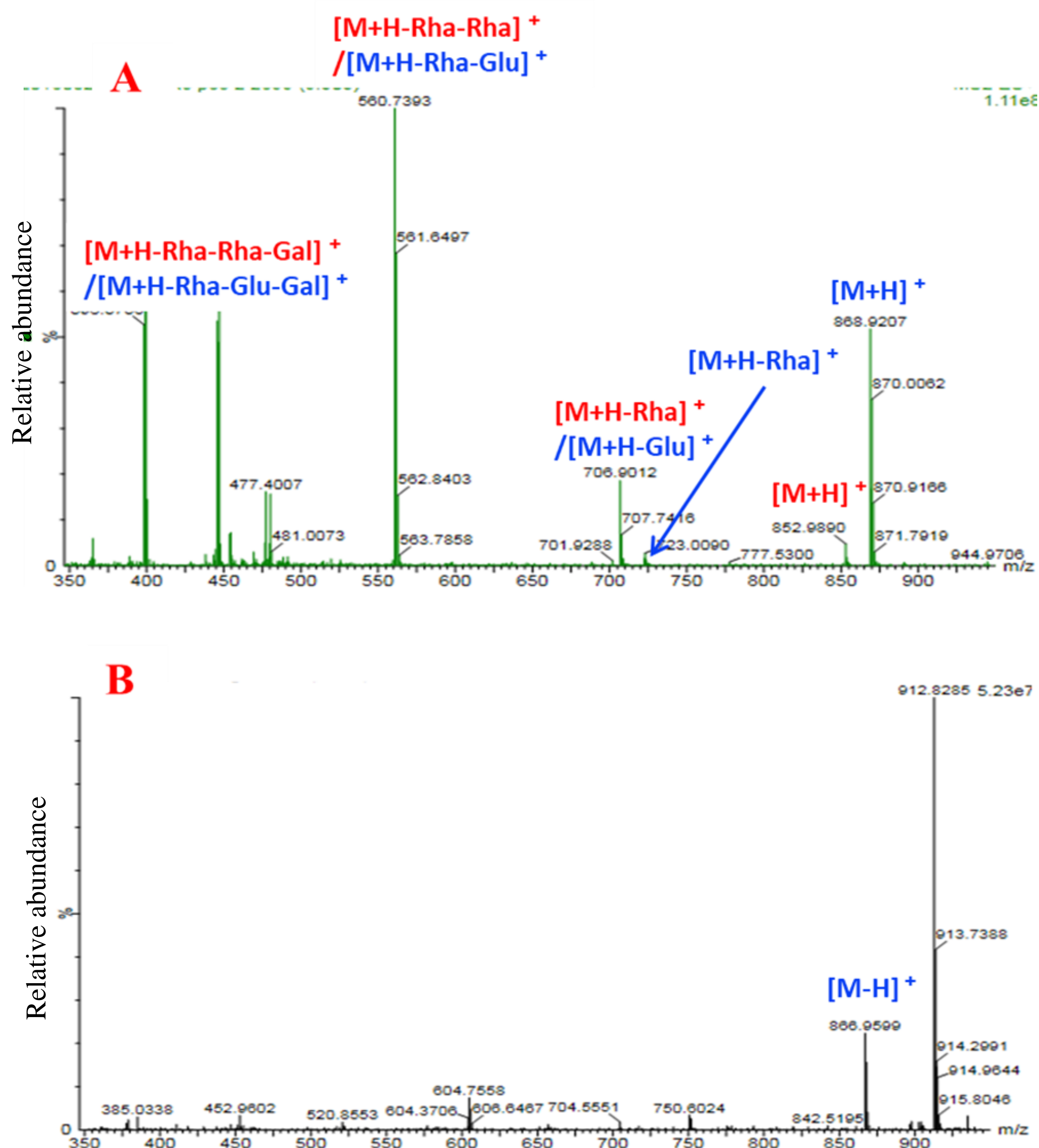


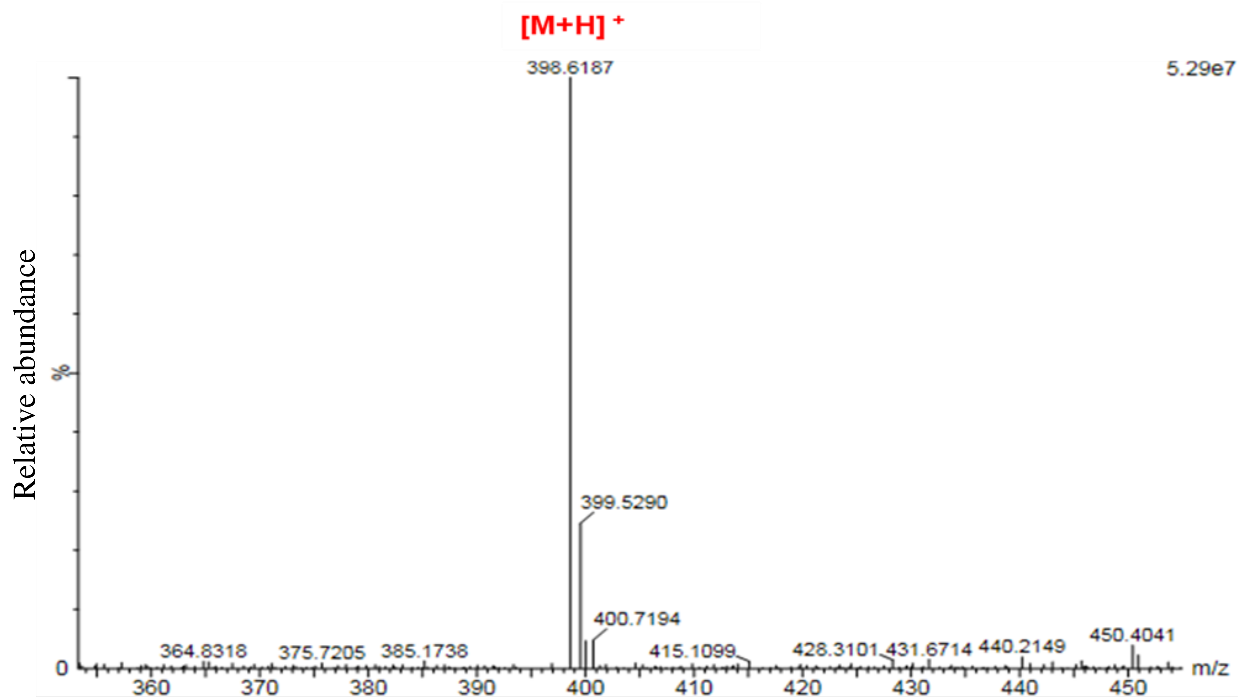

**Appendix VI:** Mass spectrum showing the ESI fragmentation pattern of methyl-dihydrojasmonate positive (A) and negative (B) ionization modes



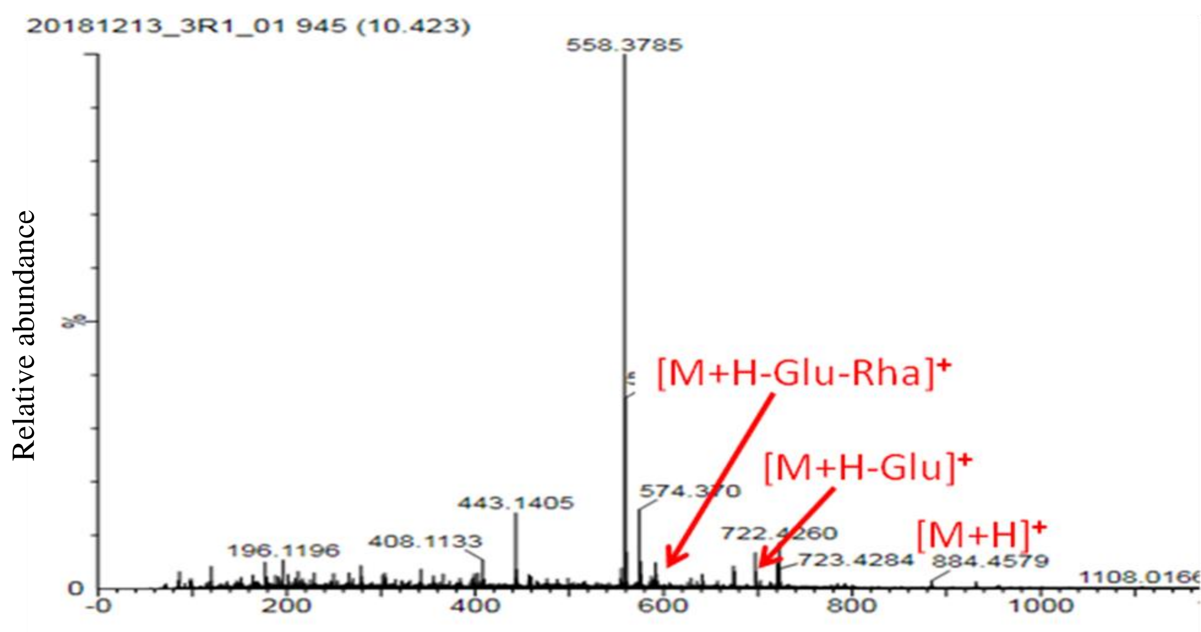

**Appendix VII:** Mass spectrum showing the ESI fragmentation pattern of solanoeclepin A positive (A) and negative (B) ionization modes



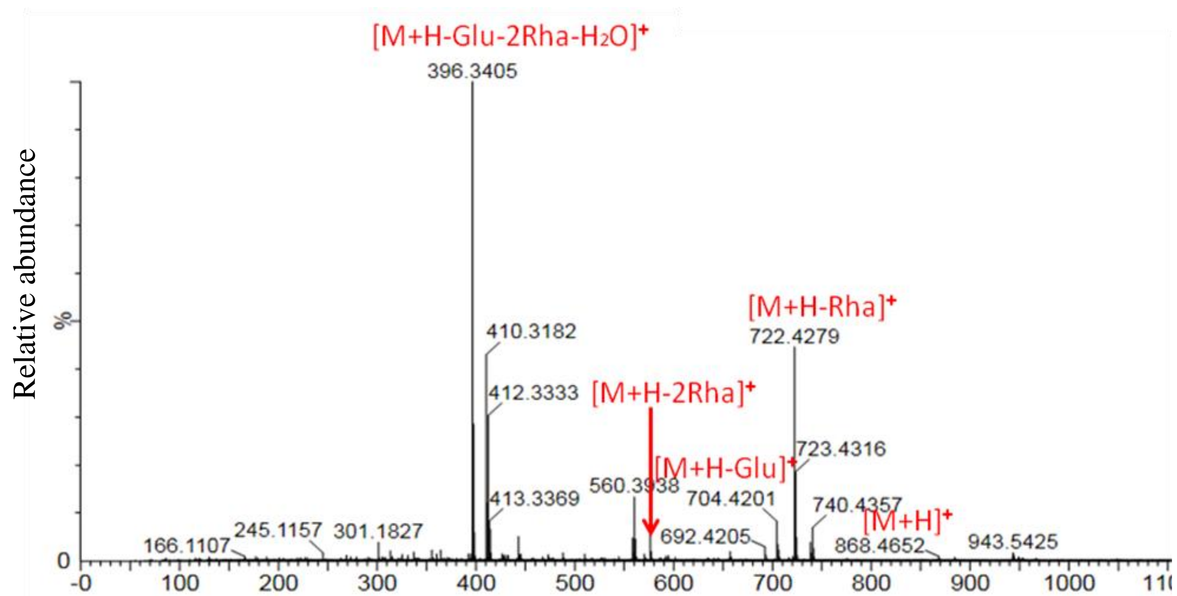

**Appendix VIII:** Mass spectrum showing the ESI fragmentation pattern of α-solanine and α-chaconine positive (A) and negative (B) ionization modes. Masses in red represent fragmentation pattern of α-chaconine while in blue represents α-solanine.




**Appendix IX:** Mass spectrum showing the ESI fragmentation pattern of aglycone solanidine positive ionization mode




**Appendix X:** Mass spectrum showing the ESI fragmentation pattern of solasonine positive ionization mode




**Appendix XI:** Mass spectrum showing the ESI fragmentation pattern of aglycone solamargine positive ionization mode

**Appendix XII:** Comparisons of PCN hatching response to ten compounds relative to PRE (positive control)

| | | Concentration (µg/ml) | | | | |
|---|---|---|---|---|---|---|
| **Treatment** | | **0.2 µg/ml** | **0.4 µg/ml** | **0.6 µg/ml** | **0.8 µg/ml** | **1 µg/ml** |
| α-Solanine | p-value | 0.108 | 0.046 | 0.000 | 0.001 | 0.390 |
| | OR (95% CI) | 2.14 (0.86-5.61) | 2.78 (1.05-8.00) | 5.25 (2.62-11.19) | 5.15 (2.02 - 14.29) | 1.38 (0.67-2.88) |
| α-Chaconine | p-value | 0.002 | 0.000 | 0.000 | 0.013 | 0.461 |
| | OR (95% CI) | 4.19 (1.69-10.90) | 5.75 (2.25-16.30) | 5.28 (2.59-11.20) | 3.44 (1.34 - 9.57) | 1.32 (0.64-2.74) |
| Solanidine | p-value | 0.466 | 0.035 | 0.497 | 0.092 | 0.389 |
| | OR (95% CI) | 1.42 (0.55-3.79) | 2.93 (1.11-8.44) | 1.32 (0.60-2.95) | 2.34 (0.89 - 6.58) | 0.74 (0.34-1.60) |
| Solasodine | p-value | 0.485 | 0.028 | 0.084 | 0.159 | 0.444 |
| | OR (95% CI) | 1.41 (0.54-3.76) | 3.07 (1.17-8.81) | 1.95 (0.93-4.25) | 2.06 (0.77 - 5.83) | 0.51 (0.22-1.13) |
| Tomatidine | p-value | 0.032 | 0.954 | 0.808 | 0.705 | 0.102 |
| | OR (95% CI) | 0.20 (0.03-0.76) | 1.03 (0.33-3.26) | 0.91 (0.38-2.11) | 0.79 (0.25-2.51) | 0.36 (0.15-0.84) |
| Phenylalanine | p-value | 0.000 | 0.767 | 0.010 | 0.008 | 0.021 |
| | OR (95% CI) | 0.12 (0.03-0.34) | 0.79 (0.14-3.86) | 0.29 (0.10-0.70) | 0.27 (0.09-0.66) | 0.09 (0.02 - 0.27) |
| Tryptophan | p-value | 0.000 | 0.192 | 0.100 | 0.003 | 0.000 |
| | OR (95% CI) | 0.06 (0.00-0.23) | 0.23 (0.01-1.64) | 0.51 (0.22-1.11) | 0.23 (0.08-0.57) | 0.06 (0.01 - 0.18) |
| Tyrosine | p-value | 0.001 | 0.195 | 0.004 | 0.010 | 0.000 |
| | OR (95% CI) | 0.06 (0.00-0.23) | 0.23 (0.01-1.64) | 0.13 (0.02-0.43) | 0.24 (0.07-0.65) | 0.03 (0.00 - 0.12) |
| Methyl-dihydrojasmonate | p-value | 0.035 | 0.006 | 0.074 | 0.039 | 0.000 |
| | OR (95% CI) | 0.31 (0.10-0.88) | 0.25 (0.09-0.64) | 0.42 (0.15-1.05) | 0.15 (0.01-0.71) | 0.09 (0.02 0.25) |
| Zeatin | p-value | 0.007 | 0.010 | 0.209 | 0.619 | 0.000 |
| | OR (95% CI) | 0.15 (0.03-0.52) | 0.28 (0.12-0.72) | 0.57 (0.23-1.34) | 0.76 (0.24-2.27) | 0.15 (0.02 0.26) |