



Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and
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Early detection of virus infections in potato by aphids and infrared remote sensing

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Credits: 30

Level: Master

Course title: Independent Project/Degree Project in Biology - Master's thesis

Course code: EX0565

Programme/education: Plant Production Biology

Place of publication: Epsilon

Year of publication: 2016

Cover picture: Infrared picture of Potato and Aphid sitting on leaf

Title of series:

Part number:

ISSN:

ISBN:

Online publication: <http://stud.epsilon.slu.se>

Keywords: Infra-red, remote sensing, PVY, Volatiles, Aphid attraction

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Abstract

Potato is one of the most important crops worldwide. In recent years, breeding and advances in crop protection have made it possible to increase potato yield and production despite decreasing area harvested. Nonetheless problems with pests are still prevalent and can destroy big parts of the annual harvest. One of the mayor problems in potatoes are viruses, due to the lack of chemical control. The Potato Leaf Rolle Virus (PLRV) and the Potato Virus Y (PVY) are the most threatening viral diseases. Both are transmitted with aphids. While it has been shown, that the persistent virus PLRV attracts aphids through altered volatiles in potatoes, this proof was lacking for the non- persistent virus PVY. Our results of aphid olfactory response have shown that the odors of potato cultivars expressing a great amount of symptoms when infected with PVY attracted significantly more aphids than the odors from virus free plants. However, only in early growing stages of the plants, PVY infected potatoes were more attractive for aphids.

Viral diseases are also transferred in vegetative propagation with seed tubers. The plants derived from these infected tubers are the source for new virus infections in the field. Unfortunately, up to now, virus detection in plants is limited to serological (ELISA) and molecular (PCR) methods, which are destructive and time consuming. Early detection of PVY infected plants and their removal from the field is the most important measure to stop/reduce virus distribution by aphids. We demonstrated that mid- infrared imaging can be used to detect virus infections in early growing stages of plants before symptoms are visible to the human eye. The diagnosis of viral infections with infrared techniques became more accurate with the age of examined plants.

Our studies indicate that remote sensing with mid infrared cameras can be used to identify PVY infected plants in a growing stage in which aphid attraction via odors occurs. Mid infrared sensing can therefore be used to detect infected plants before aphids arrive in the field.

Popular summary

Viral diseases in plants cause yield loss and reduction of yield quality in most agricultural crops. In potatoes (*Solanum tuberosum*) the Potato Leaf Roll Virus (PLRV) and the Potato Virus Y (PVY) are the most damaging viral diseases. The most important way of spreading plant viruses are aphids. It had been shown, that PLRV infected potatoes attract more aphids via plant volatiles than healthy plants. In this study it was shown, that also PVY infected potatoes attract more aphids than healthy plants. However this is only the case when potato plants are young and for potato cultivars which show strong symptoms when infected with PVY. Once the plant is infected with PVY, the virus is also transferred with seed tubers to the next generation of plants. The new growing plants contain the virus right from the beginning and form the source for virus spread with aphids the next year. The detection of viruses in plants is time consuming and expensive. A fast and less costly way of detecting viruses in the plant could be the use of infra-red cameras. This study shows that PVY can be detected in growing potato plants with mid infrared techniques before symptoms are visible to the human eye. Further is it possible to detect the infections in a growing stage in which aphid attraction occurs. So the use of infra-red images could help to eradicate infected plants from the fields, before aphids are attracted and can spread the virus.

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1. Outline

This work is twofold. It will first be evaluated if Potato Virus Y (PVY) infected potato plants attract aphids via volatiles. Secondly it will be investigated if mid infrared imaging is a potential method of diagnosing PVY infections in potatoes before visual symptoms occur. A schematic overview is given in figure number 5.

2. Introduction

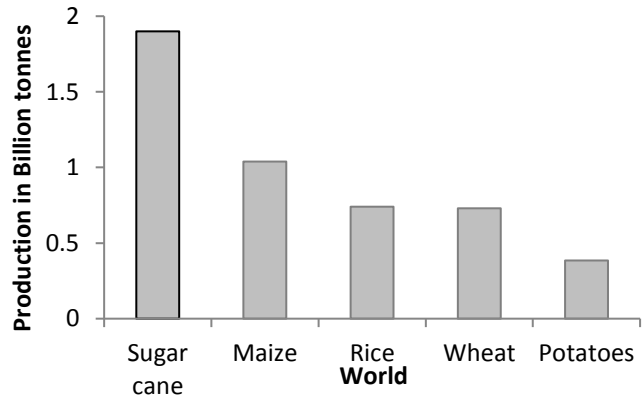
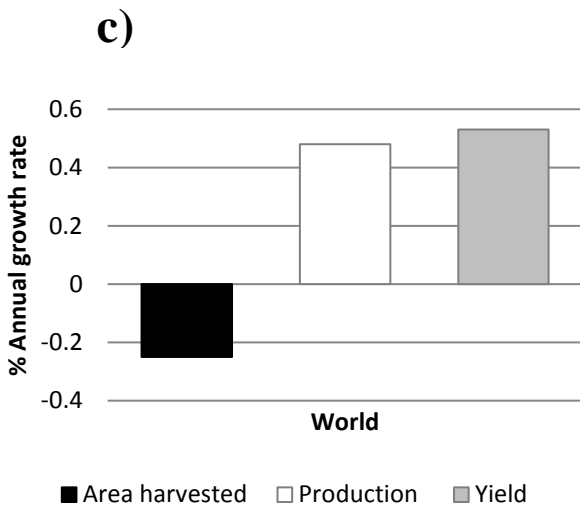
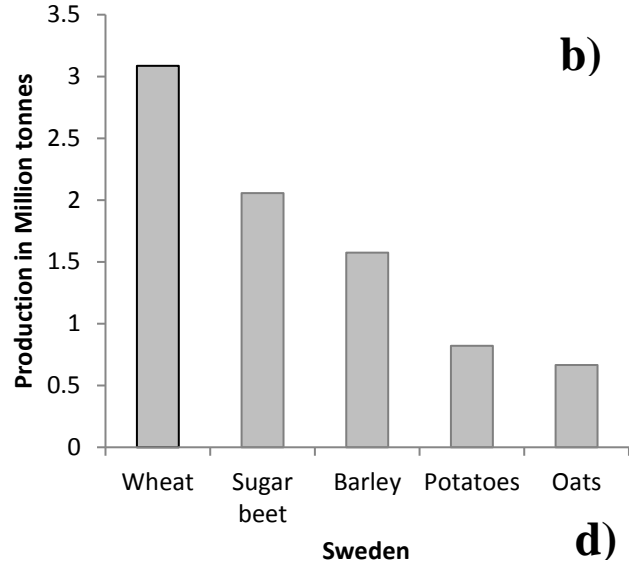
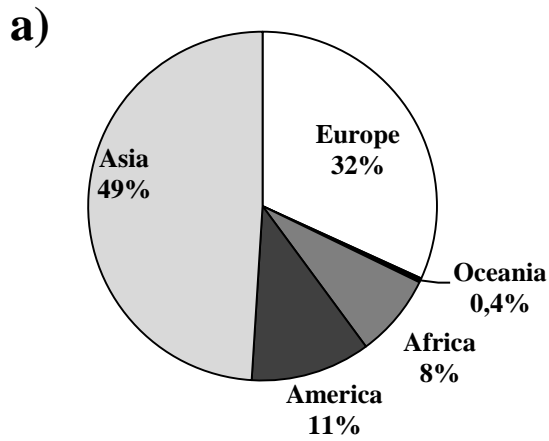
2.1) Potato production

Potato (*Solanum tuberosum*) (L) is one of the five most important food crops in the world (Oerke, 2006) with 400 million tonnes harvested in 2014 (FAO 2016) (Fig. 1d). In Sweden potato is the fourth most produced crop with approximately 800 thousand tonnes produced in 2014 (FAO 2016). It is grown worldwide, with the majority being cultivated in Asia and Europe (FAO 2016). In the last ten years, non- developed countries have overtaken the developed world in potato production (Fig. 1a). Over the last 50 years, the area on which potatoes are grown worldwide has been decreasing. However yield and production are increasing (Fig.1c), showing that breeding efforts and protection methods have increased the potatoes' resilience against pests and the yield per plant.

But despite these improvements, problems- especially with pests- are still a big issue. Without crop protection 75% of an annual harvest can be lost due to infestations of weeds, animal pests and pathogens (bacteria, chromista, fungi and viruses). The actual loses with applied crop protection range from 24% in Europe to 50% in Africa (Oerke, 2006).

As potatoes are propagated vegetatively with tubers, they accumulate pests. And with successive replanting the amount of pathogens in the daughter tubers increases from year to year, leading to lower yield and quality, so called "running

out”. Therefore many countries have established seed certification programmes to ensure clean seed potato production.



2014; b) Top five crops in Sweden 2014 ; c) Area, yield and production changes worldwide between 1961 and 2014; d) Top five crops worldwide 2014; Data source: FAO Stat 2016

2.2) Seed potato production

The two best known certification programmes are the European Scheme (UN/ECE) and the North American Scheme implemented in Canada and the USA. Both are similar in the process, but contain differences in some steps. Many other

countries follow these schemes to produce seed tubers. The main aims of seed potato programmes are to produce genetically pure (true type) and pest and disease free seed tubers (EU 2016).

In most programmes plantlets are produced from meristem cuttings. Apical meristems are often virus free and can be used *in vitro* to produce virus free plants even from infected material (Kassanis, 1957). Out of these virus free plants, basic seed tubers can be produced. The basic seedlings are then grown in the field to produce reasonable amounts of seed tubers for the ware potato production. It is expected, that tubers will again acquire pests when they are planted under uncontrolled conditions in the field. Therefore, the amount of generations in the field after meristem cultures is limited. In North America, five field generations are allowed for daughter tubers of meristem cuttings. While in Europe up to 10 generations are permitted (Gutbrod and Moseley, 2013). After these generations in the field, new meristem cuttings have to be done to ensure pest free material. For each field generation and their special purpose, the level of pests permitted in the seed tubers varies. For the first generation after the meristem cuttings, as well as for tubers intended for the next generation of seed tuber production, 0% infected tubers are allowed. Later on, if the potatoes are intended for field production, an infection rate of up to 10% is tolerated (Ragsdale et al., 2013). Post-harvest serological tests are carried out to check if generations exceed the claimed amount of pests. If they do, they are transferred into higher generations, where more pests are tolerated. This means, that “generations” in seed potato production does not necessarily coincide with “years in the field”, but is more a mean of measuring the amount of pathogens in the tuber. If the amount of infected tubers exceeds all limits, they are discarded (Gutbrod and Moseley 2013).

The ability to detect and eliminate diseased plants is crucial for the success of seed potato production. Besides fungal diseases like Late Blight (*Phytophthora infestans*), virus detection is particularly important in seed potatoes, as they account for the biggest potential loss deriving from pathogens (Oerke, 2006), due to the lack of curative chemical control of virus infections.

2.3) Potato Virus Y

On a global scale the Potato Leaf Roll Virus (PLRV) is more dangerous, while in Sweden Potato Virus Y (PVY) is more prominent (Sigvald, 1987). PVY belongs to the genus of *Potivirus*, one of the six genera in the family Potyviridae. It occurs in mainly three different strains:

- The ordinary strain: PVY^O
- Tobacco venal necrosis strain: PVY^N
- And the stipple streak strain: PVY^C

Besides these main strains several sub- or smaller strains can be found (e.g.: PVY^Z, PVY^{N-} Wilga etc.) (Blanco-Urgoiti et al 1998). Some of the sub strains were formed by recombination of the main strains, like PVY^{NTN}, which is a hybrid between PVY^O and PVY^N and is classed as a subgroup of PVY^N (Boonham et al., 2002, Glais et al., 1998) (Fig. 2).

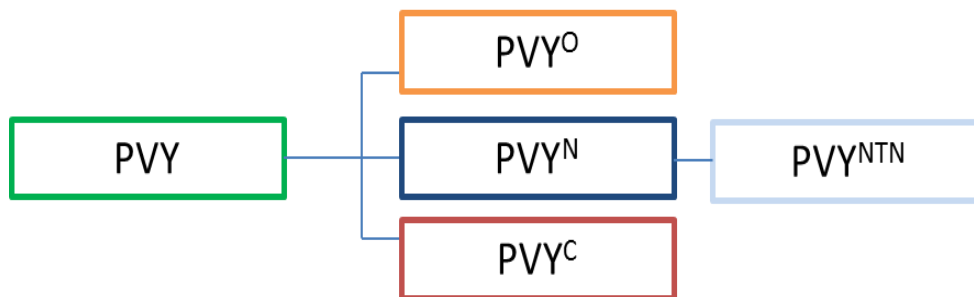


Figure 2: Pedigree of main strains of PVY

2.4) Symptoms of PVY

Each of the strains has different symptoms. PVY^O provokes a mosaic pattern on the leaves, leaf drop and stem necrosis (Fig. 3d). PVY^N attacks mainly tobacco plants and causes similar, but milder symptoms than PVY^O in potato. Sometimes the infection with PVY^N can even be asymptomatic, depending on the potato cultivar. PVY^C induces mild mosaic patterns or stipple streak. PVY^{NTN} causes Potato Tuber Necrotic Ringspot Disease (PTNRD): necrotic rings on the tubers

which can deepen into the tissue during storage and make tubers unmarketable (Fig. 3a). Due to that, PVY^{NTN} has evolved into the economically most important strain in potato production (Brunt, 2013). However, not only the virus strain determines the development and severity of the symptoms, but also the potato cultivar, as well as environmental factors are of importance. In fact, some potato cultivars show fewer symptoms than others. The yield reduction however remains the same even though symptoms on the aerial plant parts are not severe or not visible at all. Therefore visible symptom expression cannot be taken as a reliable indicator for infection (Hane and Hamm, 1999; Weidemann, 1988).

As potatoes are propagated vegetatively, viruses accumulate in the tubers and infest new deriving plants immediately. If the plants suffer from this so called secondary infection of PVY, they are often stunted and have wrinkly leaves (Kerlan, 2006) (Fig. 3b). As plant immune defence is not yet established in young plants, these infections are more severe than primary infections, derived from infections during growth (Sigvald, 1985). Uninfected potato plants can be inoculated with PVY mechanically or through damages on the plant. But the most important way of primary infection is vector transmission with aphids.

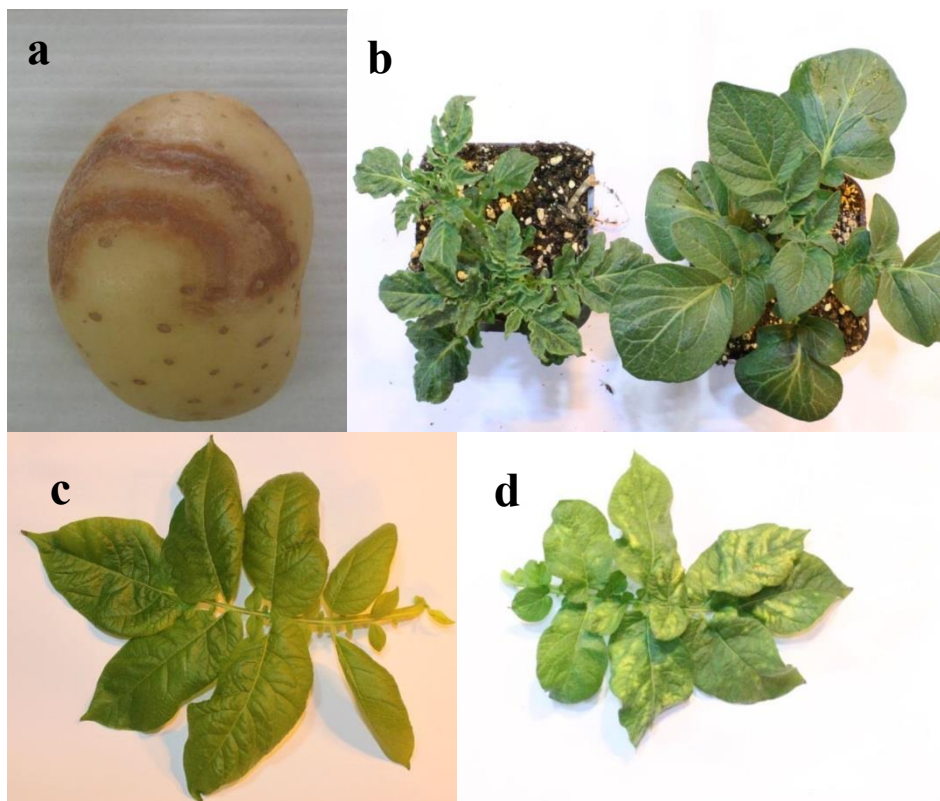


Figure 3: Symptoms of different PVY strains; a) Potato Tuber Necrotic Ringspot Disease (PTNRD) caused by PVY^{NTN} (Licence: Wikimedia commons); b) Infected (left) and uninfected (right) potato plants, cultivar King Edward at the same age; c) Healthy leaf of cultivar Solist d) Infected leaf of cultivar Solist

2.5) Aphids as virus vectors

All Strains of PVY can be carried via aphids. Some isolates of PVY^C however need simultaneous infections with PVY^O or PVY^A to be transmitted by aphids (Kerlan, 2006).

PVY spread by aphids occurs in a non- persistent manner, meaning that the virus only stays and survives within the vector aphids for some minutes up to one hour. In contrast to that, persistent viruses (like PLRV) can be retained in the aphid and be infectious for days up to the whole lifespan of the aphid. Non- persistent viruses stay in the stylet part of the aphid and do not enter the actual body, whereas persistent viruses enter the aphid and stay, move and even proliferate in

the insect. Persistent viruses are therefore also referred to as circulative, while non-persistent ones are non-circulative (Fig. 4) (Nault, 1997).

Many aphid species conduct brief and superficial probe pricks to ensure they are located on the right host, before they pierce deeper to reach the phloem, which is their normal feeding side. This superficial probing normally only lasts for seconds and does not reach deeper than the epidermis of the plants. However this small time span and depth is sufficient to acquire non-persistent viruses from plants (Nault, 1997; Ng and Perry, 2004; Pirone and Harris, 1977). If the penetration goes deeper, the acquisition rate of non-persistent viruses drops rapidly (Nault and Bradley, 1969). The short probing phase is the crucial phase for inoculation with non-persistent viruses.

More than 70 aphid species are able to spread PVY (Sigvald, 1984; Radcliffe and Ragsdale, 2002; Pelletier et al., 2012). However, not all of them occur in all regions of Sweden and not all of them are equally efficient in transmitting the virus. The Green Peach Aphid (*Mysus persicae*) (Sulz) does colonize potatoes and many other species out of the Solanaceae family and is the most efficient transmitter of PVY with –when carrying the virus- 26% of its probed plants infected (Sigvald, 1984). Even though it occurs only in southern Sweden and even there in smaller numbers it is one of the most important vectors of PVY in Sweden. The most abundant aphid in Sweden which can transmit PVY is the Bird Cherry Oat Aphid (*Rhopalosiphum padi*). Even though the transmission efficiency of *R. padi* is much lower than *M. persicae*'s (1-7% of probed plants infected) (Sigvald, 1984) it plays the most important role in spreading PVY in Sweden, due to its total number and occurrence in all regions of Sweden (Sigvald, 1987). *Rhopalosiphum padi* does not colonize potato, but due to spring migration from their primary host - the bird cherry tree (*Prunus padus*)- to its secondary hosts - cereals from the *Poaceae* family (wheat, barley, oats and pasture grasses)-, *R. padi* might land on potato plants as well, while in search of actual secondary hosts.

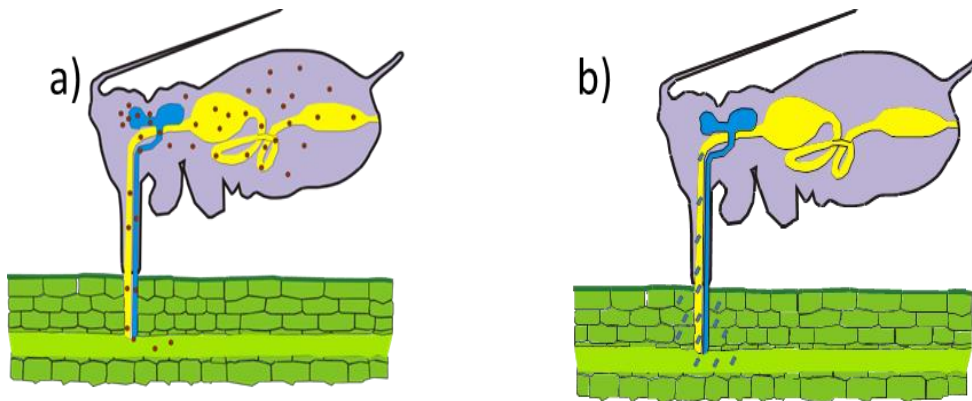


Figure 4: Virus-vector relationships of (a) persistent PLRV and (b) non-persistent PVY in aphids. Red dots: persistent virus particles, Blue stripes: non-persistent virus particles. Modified after: Edgar Schliephake

2.6) Aphid host detection and location

To find proper hosts, aphids use a series of cues. First of all they use visual cues, colours as well as shapes and structures to find potential host plants (photo taxis). It has been shown, that most aphids are attracted to yellow colours (Fereres et al., 1999; Fereres and Moreno, 2009), but *R. padi* shows higher attraction to green colours (Archetti and Leather, 2005). Photo taxis is used to find plants in general and not to land on, for example, rocks. A host/ non-host discrimination does not occur with photo taxis (Powell et al., 2006). The final host recognition occurs after landing on a plant with the already mentioned brief probing which is normally restricted to the epidermis. However, between the recognition of plants via colours and the landing on and probing of the plant, plant odours can be perceived with sensory organs in the antennae of aphids (Park and Hardie, 2004). They serve as the first actual indicator for host/ non-host discrimination and influence the landing behaviour (Nottingham and Hardie, 1993).

These plant odours have several functions. As a measure of plant-plant communication, plants release and receive volatile organic compounds (VOCs). These compounds and their composition change when the plant is under stress. This change can be detected by neighbouring plants, which in reaction to it, can brace themselves, for example, against herbivory insect attacks (Hare, 2011). Also

plants can attract herbivory natural enemies with volatiles to help them fend off attacks of plant eating insects (Vucetic et al., 2014). But besides these positive effects of VOCs for the plant, aphids and other plant feeding insects use olfactory cues to find their host plants (Nottingham et al., 1991).

It has been shown, that plants, infected with persistent viruses, attract aphids with changed olfactory cues and make their retention time on the plant shorter, to increase virus spread (de Vos and Jander, 2010). The Barley Yellow Dwarf Virus and PLRV – both persistent- attract *M. persicae* and *R. padi* in potatoes and barley respectively, via altered VOCs from the plant (Eigenbrode et al., 2002; Jiménez-Martínez et al., 2004; Medina-Ortega et al., 2009).

It has been unclear, if non- persistent viruses attract aphids to infected plants via VOCs in the same way persistent ones do. To answer the question why *M. persicae* and *R. padi* are so successful in spreading PVY in Sweden, we investigated olfactory cues to see if also non- persistent viruses attract aphids with volatiles from infested potato plants.

2.7) Thermal imaging in agriculture

For seed potato growers the knowledge about aphid attraction to virus infected plants can give important information and implications for virus management strategies; like application time points of insecticides against the virus vectors. However, it is unlikely, that virus spread can be avoided completely. Therefore it is important to detect virus infections in the field as early as possible and eradicate infected plants to prevent the virus from spreading further.

Unfortunately, virus symptoms visible to the human eye occur with some latency time. And between the point of virus infection of a plant and the occurrence of symptoms, the virus might have already spread to other plants. Common techniques for virus detection, like serological methods (ELISA) or molecular methods (PCR) are destructive, time-consuming, labour intensive and therefore expensive. A non-destructive and fast method of virus detection could be the use of mid wave infrared remote sensing. Virus infected plants exhibit different

patterns than uninfected plants on infrared images (Chaerle et al., 1999). These differences could help to detect virus symptoms on plants, before visible to the human eye.

Infra-red remote- sensing has been used for several years in agriculture to determine the state of plants and canopies. Thermal emissions can give insight into the physiology of plants and detect stresses like water, heat or nutritional stress (Hunt and Rock, 1989; Tucker,1980;Buitrago et al., 2016; Gómez-Bellot et al., 2015). In breeding, monitoring biomass, phenology and physiological conditions of plants and canopies with near infrared techniques (Peñuelas and Filella, 1998) helped to ease and accelerate selection processes. Also seeds can be tested for their viability (Kim et al., 2013) and diseases derived from fungi (Oerke et al., 2011), bacteria and viruses (Chaerle et al., 1999) on plants can be detected before visible symptoms appear. A weak spot of infrared based observation methods is that a lot of factors influence the reflective patterns of plants. Abiotic and biotic stresses like other diseases, nutrition and water status can mask the effect of the factor one is actually looking for (Chaerle and Van Der Straeten, 2000).

Until recently research and applications for remote sensing had focused on the visible (390 to 700 nm) and near infrared spectra (0.75–1.4 μm). But latest findings have shown that also mid infrared (3- 8 μm) and long infrared wavelengths (8-15 μm) reveal interesting and important facts about plants (Ullah et al., 2012; Kim et al., 2013; Oerke et al., 2011).

Virus detection was up to now tried with fluorescence methods, in the far infrared spectrum and with near infrared wavelengths. Different viruses (Tomato Mosaic Virus, Soybean Mosaic Virus) in different species could be detected (Chaerle & Van Der Straeten ,2000; Chaerle et al., 1999; Jinendra et al., 2010; Xu et al., 2006). Though for potatoes, as well as for the most important virus in Swedish potato production – Potato Virus Y (PVY) - (Sigvald, 1987), no infrared method has yet been established. PVY is one of the most damaging viruses, with reported possible yield losses between 40- and 70% (Blanco-Urgoiti et al., 1998). The possibility to detect this virus in the field at a very early stage of plant growth would facilitate seed potato production in Sweden and worldwide immensely.

As viruses affect multiple physiological and morphological parameters in the plant, the reason for altered thermal reflectivity can have several causes (Culver and Padmanabhan, 2007). The focus of this study was to see if it is possible to discriminate between healthy and PVY infected plants with thermal images. The underlying reasons for the occurring differences were not evaluated.

2.8) Aim of study

It is going to be evaluated if aphids are attracted to PVY infected potato plants via volatiles. This knowledge can help to prevent the spread of PVY with aphids. However total control will never be possible. And therefore in a second step it is going to be tested if it is possible to detect secondary infections of PVY in potato via mid infrared images at an early stage of growth.

3. Material and Methods

Study setup and Plant material

The study consists of two different experiments (Fig. 5). For both experiments the same two potato cultivars were used

- Solist (*Solanum tuberosum* L. cv. Solist): A variety expressing only mild symptoms when infected with PVY
- King Edward (*Solanum tuberosum* L. cv. King Edward): Expressing strong symptoms when infected with PVY

The potato tubers were collected from farmers in Sweden and checked for PVY infections with ELISA tests. A single eye from a tuber was planted per pot (9x9x7cm). Potting soil was fertilised with NPK fertiliser Yara Mila® (11% N, where of 4,4% is nitrate and 6,6% is ammonium; 4,6% P whereof 3,5% are water-soluble; 17,6% K; 1,6% Mg; 10% S, 0,25 Mn; 0,08% Fe; 0,05% B; 0,03% CU) (120g fertiliser per 100 L soil). After one week, when the eyes started to sprout, the plants were re-potted into two litre pots. The plants were watered once per day. Light was artificially provided with gas discharge lamps in the blue and

yellow spectrum for 16 hours a day. Temperature was always at 20 ± 2 ° C. The plants were not inoculated artificially with PVY. All infections were secondary infections coming from the tuber.

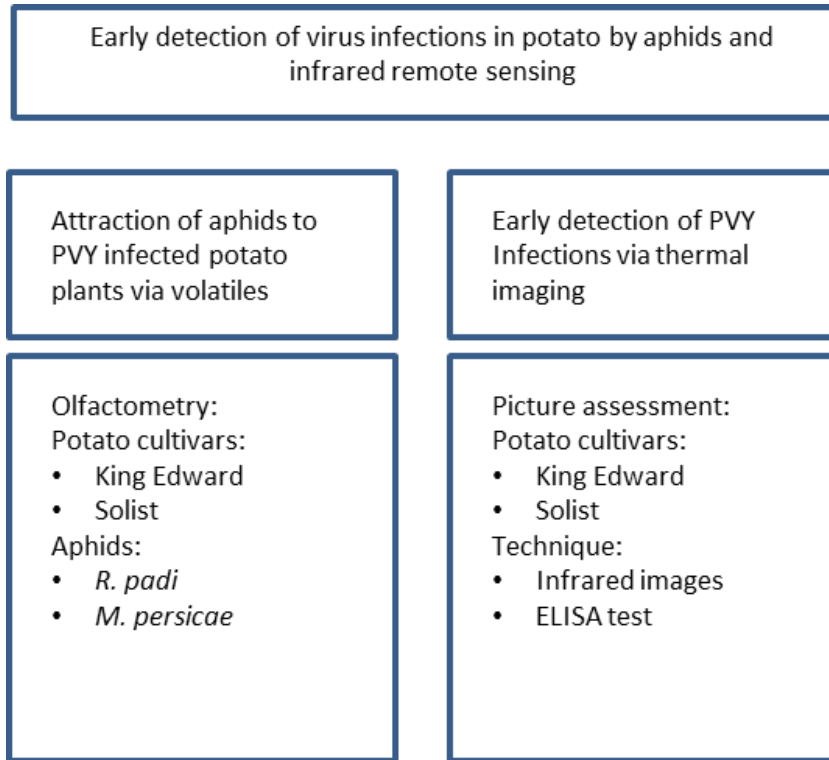


Figure 5: Schematic setup of the studies and materials and methods of the individual parts

3.1) Aphid attraction to PVY infected potato plants by volatiles

Aphid material

The aphid species used were Bird Cherry Oat Aphid *Rhopalosiphum padi* (L) and Green Peach Aphid *Myzus persicae* (Sulz). They were reared in cages under controlled conditions. Light was provided from florescence lamps 16 hours a day. Temperature was 18 and 20 ± 2 ° C for *R. padi* and *M. persicae* respectively with relative humidity of 50%. *R. padi* was fed on barley (*Hordemum vulgare*) (L), while *M. persicae* was fed on rapeseed (*Brassica napus*) (L).

Olfactometry

Two way olfactometry was used to determine the preference of *R.padi* and *M. persicae* to odours of PVY infected plants. Fourteen to 21 day old plants of the cultivars King Edward and Solist were used. Infection of planted tubers was assumed when mother plants of the tubers had positive ELISA results. Infected King Edward plants showed virus symptoms while at that stage no symptoms were visible in Solist. One arm of the arena was connected to a cage containing an infected plant the other one to a cage containing an uninfected plant (Fig. 6). The side of uninfected and infected plant cages was changed in every setup to ensure randomisation. Airflow was established by a vacuum pump with 0.8-L per minute. The surrounding temperature was always above 20° C. Artificial light was set at 60 $\mu\text{mol m}^{-2}\text{s}^{-1}$ with fluorescent lamps and possible effects of natural sunlight were blocked with curtains. Per potato variety, 20 insects of each aphid species were tested. All aphids used in the olfactometry experiment were wingless.

Before the first recording, the insect was given ten minutes to acclimatise. The visits of the aphid to the different sides in the arena (arm with odours from infected plants, neutral position or arm with odours from un- infected plants) (Fig. 6) were recorded manually every three minutes for half an hour. Three minutes is the time an average aphid needs to crawl once around the arena (Pettersson, 1993). In total ten recording points were taken per aphid. In the neutral zone of the olfactometry arena, odours from both arms blend, and the aphid might perceive both of them. Therefore insect behavioural studies do not consider visits to the neutral position as “choice” and do not include them in their analysis (Ninkovic et al., 2011; Pettersson et al., 1994; Quiroz and Niemeyer, 1998). Also in this study only visits to the arms with odours from infected and non- infected plants were analysed. Visits to the neutral zone were not taken into account.

Data analysis was done with SPSS (Version: 22). As the visit data was not normally distributed, the non-parametric Wilcoxon signed rank test was used (Ninkovic et al., 2011). Visit frequencies were considered significantly different, when $p < 0.05$.

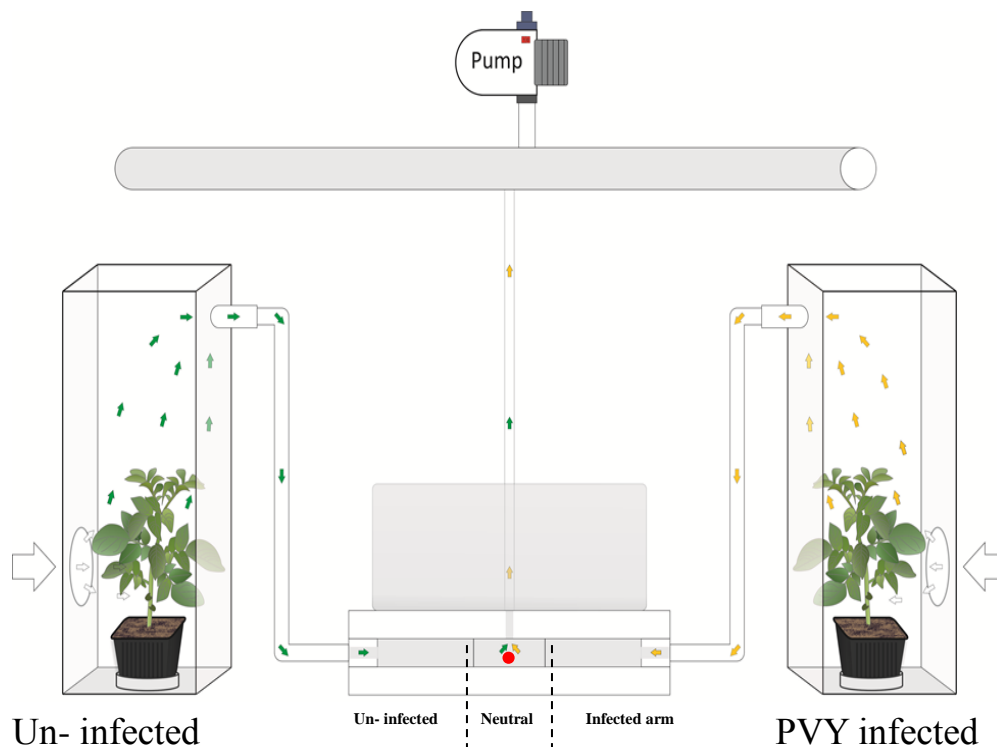


Figure 6: Olfactometry setup. Green arrows: Odours from uninfected potato plants; Yellow arrows: Odours from infected potato plants; Red point: Starting point of aphid. Modified after: Dimitrije Markovic

To investigate the preference of aphids in different plant age groups, an attraction index (AI) was calculated (Modified after the work of Dekker et al., 2006 and Tipping et al., 1987).

$$AI = \frac{T - C}{10}$$

With T= Visits to arm with odours from infected plants, C= Visits to arm with odours from un- infected plants, and 10 as the total amount of visits possible. An attraction index of 1 indicates that aphids were exclusively attracted to volatiles of PVY infected plants. An index of -1 indicates that aphids were exclusively attracted to volatiles from healthy plants. An index of 0 indicates equal attraction of aphids to healthy and virus infested plants.

To compare the attraction indexes between plant age groups a Mann-Whitney U test was used (SPSS; Version 22). Differences between age groups were considered significant at $p < 0.05$ level (single tailed).

3.2) Early detection of PVY Infections via thermal imaging

Camera

The camera used for thermal imaging was a SC7600 (FLIR Systems, Wilsonville, OR, USA) with a resolution of 640×512 pixels. Its spectral range was 2–5 μm . The sensor was cooled using an integrated Stirling cooler. The system sensitivity was 18 mK at 25 °C. One final image consisted of the average of 100 frames taken, to reduce noise disturbance and achieve clearer pictures.

Thermal imaging

Pictures of plants aging between two and 18 days were assessed. As the focal depth of the camera was not very high, plants were grouped according to their height. Pictures were taken of the plants of same height. The camera was refocused for each group to get clear and sharp pictures.

Images were assessed visually for virus patterns by three different observers. In total 1390 images were taken. However due to the weak focal depth, only 375 were constituted a sufficient quality by all three observers to be used in the final evaluation.

Observers could classify the pictures into three different groups: Virus infected, healthy and uncertain. The three different groups were given numbers to facilitate statistical analyses.

The accuracy with which the infection state could be determined on thermal images, was calculated with the formula

$$ACC = \frac{\sum True\ positive + \sum True\ negative}{\sum Total\ population}$$

With “true positive” being plants infected with PVY and judged as such and “true negative” being plants not infected with PVY and judged as such.

Plants were grouped according to their age to be able to see how the accuracy of determination changes over time.

Double Antibody Sandwich ELISA

Samples for DAS (Double Antibody Sandwich) ELISA tests were taken from two parts of the potato plants, one branch from the top part of the plant, and one from the lower part. The branches were squeezed and the sap was mixed with sample buffer, and deep-frozen for at least one night before running the ELISA. Pure sample buffer was used as negative control. The positive control was PVY protein, the Antibody used for coating and conjugate was PVY IgG (mono cock), all provided by Bioreba AG (Switzerland). The ratio of antibody to coating buffer and conjugate buffer was 1/1000. The protocol for ELISA is given in the appendix. ELISA plates were read by a Thermo Scientific Multiscan FC at a frequency of 405 nm. Final ELISA test results were the average of the absorptions of the two samples taken per plant. Absorptions less than two times of the negative control were classed as PVY free. Absorptions double the one of the negative control were classed as infected. Absorptions five times the one of the negative control were classed as highly infected. The absorption values used were taken 24h after inoculation with the colour agent.

Pearson correlations between the image observation results and the ELISA results were calculated with SPSS (Version 22).

4. Results

4.1) Aphid attraction to PVY infected potato plants by volatiles

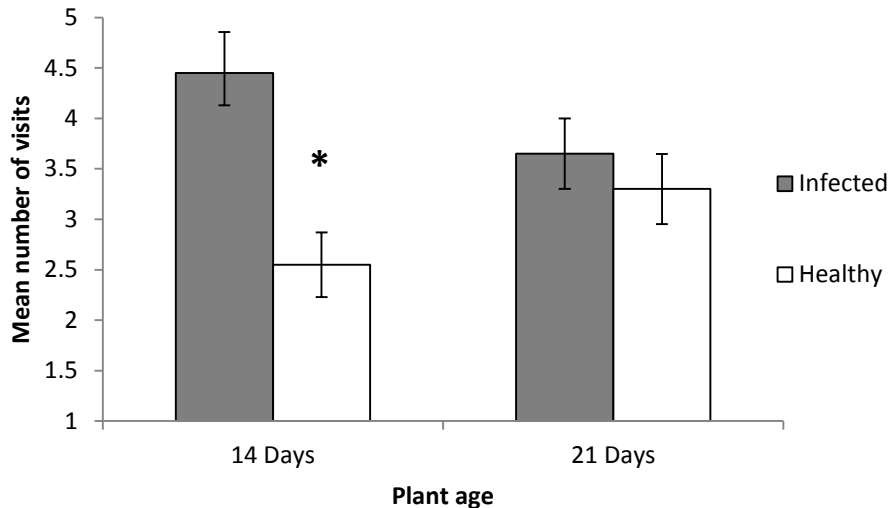


Figure 7: Mean number of visits of *R. padi* to channels with VOCs from PVY infected and healthy plants of the cultivar King Edward at different plant ages. (*= $P < 0.01$; Wilcoxon signed-rank test). Error bars show Standard Error.

Volatiles of two weeks old plants of the cultivar King Edward attracted *R. padi* significantly more to plants infected with PVY than to healthy plants ($n=20$, $Z = -2.74$, $p = 0.006$). For three weeks old plants *R. padi* was equally attracted to infected and non-infected King Edward plants ($n=20$, $Z = -0.68$, $p = 0.496$) (Fig. 7).

Myzus persicae showed a similar pattern of attraction as *R. padi* for King Edward plants. Even though the differences in attraction by odors to the infected channel and the healthy channel are not significant ($n=20$, $Z = -1.86$, $p = 0.063$) a strong tendency to prefer the volatiles of young infected plants can be seen. Comparable to *R. padi*, the attraction to infected plants

drops within one week to an equivalent attraction to healthy and infected plants ($n=20$, $Z= -0.62$, $p= 0.536$) (Fig. 8).

On average between 2.95 and 4.15 visits were counted to the neutral zone for both aphid species on King Edward (data not shown).

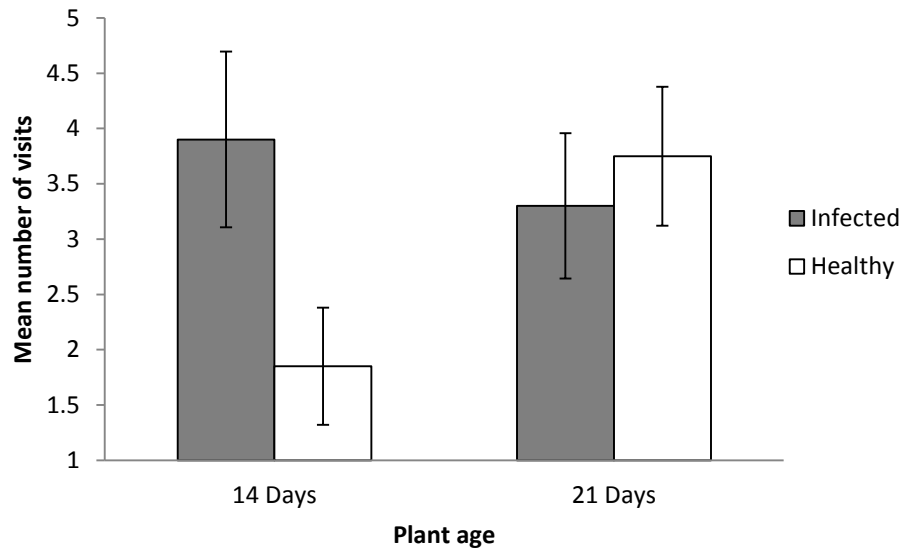


Figure 8: Mean number of visits of *M. persicae* to channels with VOCs from PVY infected and healthy plants of the cultivar King Edward at different plant ages. Error bars show Standard Error.

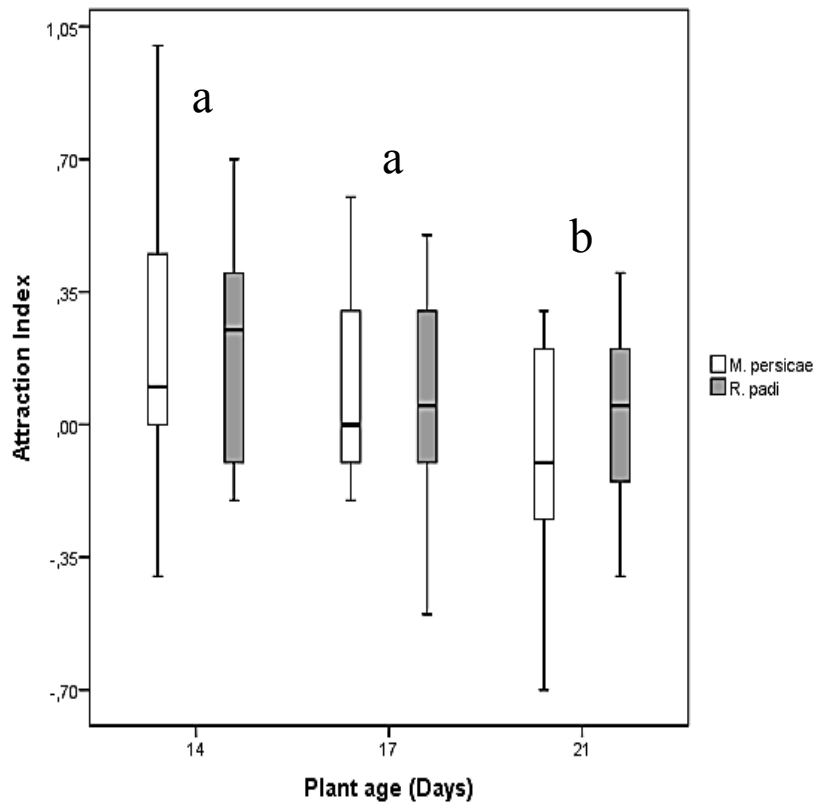


Figure 9: Attraction Index ($AI = \frac{T-C}{10}$) (T= Visits to infected side, C= Visits to healthy side, 10 representing the total amount of visits possible) for *R. padi* and *M. persicae* to King Edward plants of different ages (days after sprouting). Letters above the bars indicate significant differences

To investigate the preference of aphids in different plant age groups, an attraction index (AI) was calculated. The AI was significantly higher for two weeks old plants than for three weeks old plants for both aphid species ($p= 0.032$ for *R. padi*, $p=0.02$ for *M. persicae*). For 2.5 week old plants no significant difference to the other age groups was found (Fig. 9). Also between the two different aphid species within the same age groups of the plants no significant difference was evident (data not shown).

However, for the cultivar Solist which does not express strong visual symptoms no significant preference to VOCs of infected plants was discovered (Fig. 10). A comparison over time was impossible for this cultivar, as the growing chamber got

infested by pests, and the potato plants got unusable. Similar to the behavior on King Edward, both aphid species visited the neutral zone on average between 3.1 and 4.2 times (data not shown).

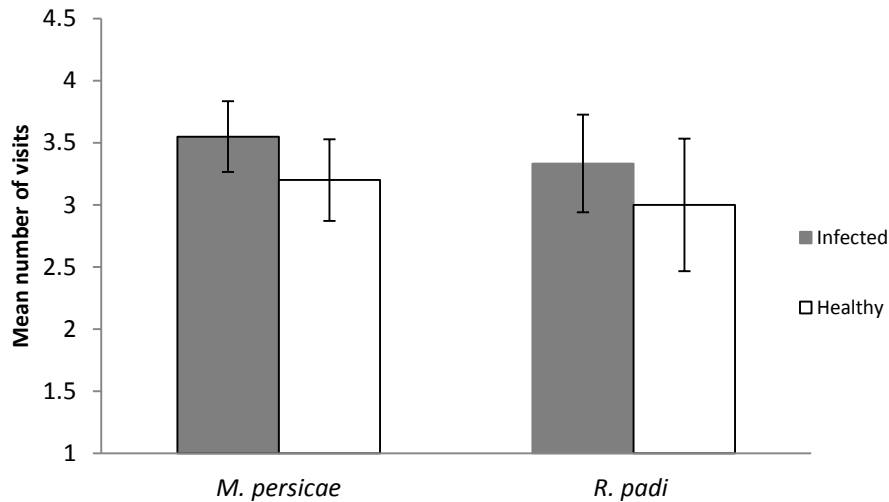


Figure 10: Mean number of visits of *M. persicae* and *R. padi* to channels with VOCs from PVY infected and healthy plants of the cultivar Solist at the age of two weeks. Error bars show Standard Error.

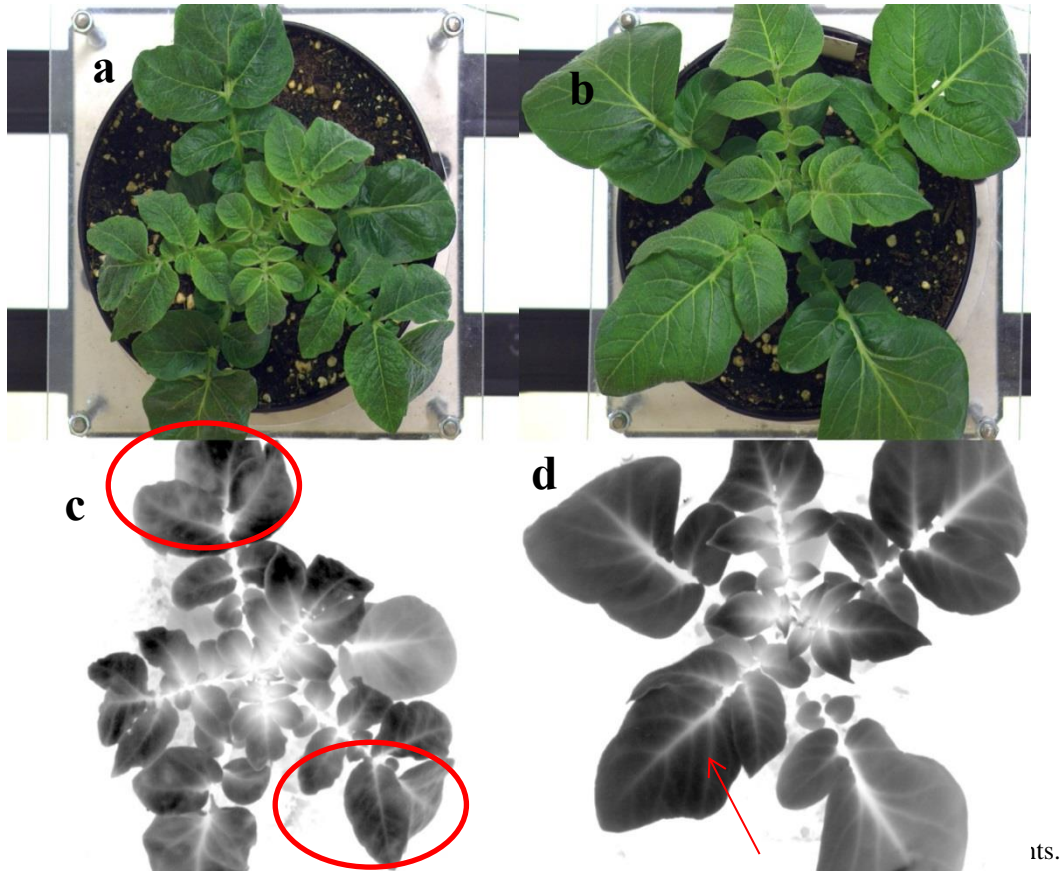
The findings let conclude that cultivar as well as the age of the plant are essential for the attraction of aphids via volatiles to PVY infected plants. Cultivars with a high expression of symptoms attracted *R. padi* while symptomless cultivars did not. For *M. persicae* the same tendency could be observed, but no significant difference was found on the 0.05 level.

However this was only the case for young plants. Older plants are equally attractive to aphids, no matter if they are PVY infected or healthy.

4.2) Early detection of PVY Infections via thermal imaging

Plants infected with PVY exhibited different properties on infrared pictures than non- infested plants. While healthy plants showed an equal distribution of thermal signatures, infected plants had scattered, unclear distribution (Fig. 11c). Further

healthy plants exhibit clear and distinct vein structures, while in infected plants veins were blurred or not visible (Fig. 11d).



A) and c) show images of the same PVY infected Solist plant. B) and d) show a healthy Solist plant. Red arrows indicate clear venal structures in image of healthy potato plants; Red circles show scattered distribution of thermal signatures in image of infected potato plant.

Infections with PVY were clearly visible in infrared images, even though no symptoms were observable with the naked eye (Fig. 11).

Table 1: Pearson's correlation between different observers (A,B,C) the average of all observers (AV) and ELISA test results. n= 375 (** significant at the 0.01 level)

	A	B	C	AV	ELISA
A	1	0.63**	0.67**	0.89**	0.53**
B		1	0.66**	0.86**	0.54**

C	1	0.89**	0.56**
AV		1	0.62**
ELISA			1

The strong correlations between the single observers displayed in Table 1, prove consistency in the judgement of the images. The correlations of the individual observers to the ELISA results were moderate. If the values from the individual observers were combined to an average value (AV), it had a stronger correlation with the ELISA results, than the individual observers.

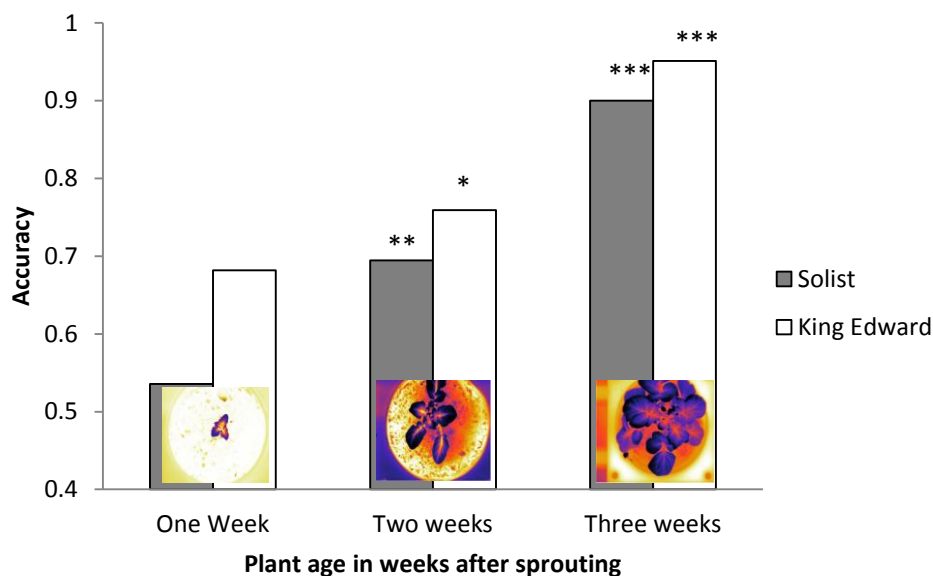


Figure 12: Accuracy ($ACC = \frac{\sum \text{True positive} + \sum \text{True negative}}{\sum \text{Total population}}$) in determination of PVY infected and healthy potato plants from thermal images sorted after plant age groups (days after sprouting; One week= 0-6 days, Two weeks= 6-12 Three weeks= 12– 18 days). (Significantly different from random choice at * =0.05 level, ** = 0.01 level, ***= 0.005 level) (n over all age groups and cultivars=375). Infrared images show plants at the respective stages.

Cultivar as well as plant age were crucial for the accuracy (ACC) with which observers were able to correctly discriminate healthy and PVY infected potato plants on thermal images (Fig. 12). In all three age groups the infection state of

King Edward can be determined with a higher accuracy than Solist's. However for the first week after sprouting the accuracy values were not statistically significant. For the second week of growth all ACC values were significant and the two cultivars adjust to each other in their accuracy values. 76% of all King Edward plants and 69% of all Solist plants could be detected correctly at this time point. In the third week of growth 95% of the King Edward plants and 90% of the Solist plants can be correctly discriminated in healthy and PVY infected.

It can therefore be concluded, that discrimination between healthy and PVY infected potato plants via thermal imaging is possible. With increasing plant age the discrimination becomes easier. Also cultivars which express more symptoms when infected with PVY are easier to judge than cultivars with only little expression of symptoms.

5. Discussion

PVY is one of the most damaging virus diseases in Swedish potato production. It is mainly spread with aphids, but once a plant is infected, the virus is also propagated with the daughter tubers. These so called secondary infections build the new threshold for virus spread with aphids in the next year. The aim of this study was to see if aphids are attracted via volatiles to PVY infected potato plants. Further the possibilities to discriminate between healthy and PVY infected potato plants with the help of infrared imaging was tested. If secondary infections can be detected early, and diseased plants are eradicated, the source of virus inoculum for aphid vectors is limited.

5.1) Aphid attraction to PVY infected potato plants by volatiles

Non persistent Viruses attract aphids via volatiles

It had previously been shown, that aphids are attracted by volatiles to plants infected with persistent viruses (Castle et al., 1998; Eigenbrode et al., 2002). We

found, that *R. padi* is also attracted by VOCs to plants of the cultivar King Edward infected with non-persistent PVY. The olfactory arm with VOCs from infected King Edward plants was visited twice as much by *M. persicae* than the arm with odours from healthy plants. However, the increased attractiveness of *M. persicae* was not significant at the 0.05 level.

Castel et al. (1998) found, that potato plants infested with persistent PLRV are more attractive for aphids than plants infected with non-persistent PVY. On these findings and on the fact that PVY has a much wider host and vector range than PLRV he postulated that the need of PVY to attract aphids in order to spread was evolutionary not as important as for PLRV. In this present study however it was shown that in absence of persistent viruses, PVY does attract aphids to infected potato plants. These findings do not contradict the ones of Castle et al. (1998) or Eigenbrode et al. (2002); they serve more as an extension, as in this study no persistent infested plants were tested against non-persistent infected ones.

Influence of potato cultivar and plant age on aphid attraction

Differences in attraction between cultivars with a high expression of symptoms and less symptomatic cultivars could be seen. Cultivars expressing more symptoms had attracted *R. padi*, while varieties with milder expression did not. It can be assumed that the virus induces a change on the level of volatiles in the same way as on the morphological level. In plants which manage to suppress visual symptoms this might be the case for volatile “symptoms” as well. To confirm these hypotheses the volatile compositions of healthy and infected plants of varieties with different levels of symptom expression have to be studied further. Ngumbi et al. (2007) showed that for the attraction to persistent infected potatoes not a single volatile compound is responsible but the altered profile of compounds. Further he showed that the total amount of VOCs released from infected plants is bigger than from uninfected ones. This might be the case for non-persistent viruses as well.

However, with increasing age of the plants, the attraction via volatiles towards the PVY infected potatoes reduced, and about one week after the first measurement

the odour from healthy plants was equally attractive for the aphids as the odour from infected plants. Normally plants develop more visible symptoms with age (personal observation). But it seems like for volatile “symptoms” it is the opposite way. It would make sense that plant viruses try to promote their spread in an early stage of plant development, because plant defence against viruses increases with the age of the plant (Sigvald, 1985).

The decrease in attractiveness of virus infected plants with age might also be due to the fact that older plants in general are less attractive for aphids than younger plants (Kennedy et al., 1950). Further, the volatile profiles of old and young potato plants are different (Agelopoulos et al., 2000) which indicates that the attraction of aphids to younger plants infected with PVY can be partly explained by these factors.

Sigvald (1987) identified *R. padi* to be the most important vector of PVY in Sweden. He accredited this importance to the fact that *R. padi* occurs in great numbers throughout Sweden and to the fact that potato is not its host plant, and so more plant visits occur. This study shows, that *R. padi* was attracted to PVY infected potatoes of a certain cultivar. This implies that the successfulness of *R. padi* in spreading PVY is not only coincidence due to the great number of these aphids, but also partly controlled by the virus itself.

Implications for seed potato production

The findings of our study imply, that aphid control in early growing stages is essential, as in this stage attraction to PVY infected plants via volatiles occurs. As potato cultivars with less symptom expression did not attract aphids to plants infected with PVY, the cultivation of these varieties might be less susceptible to virus spread. With a deeper knowledge of the mechanisms behind vector attraction to PVY infected potatoes the spread of the virus could be reduced. The timing of insecticide applications could be coordinated with the plant stages in which aphid attraction occurs. Further, if the volatile compounds or compositions which attract aphids to infected plants would be known, it could be used in aphid traps.

Proposals for future olfactometry studies

The present studies were conducted exclusively in the Lab and with wingless aphids. The effects in the field as well with winged aphids- which have different properties in the odour perceiving antennas (Hardie et al., 1994)- have to be studied. In the presented study *M.persicae* showed a slightly higher attraction index to volatiles of healthy plants three weeks after planting. It would be interesting to see, if this trend continues with further aging of the plant or if it stabilizes around a random distribution.

Between 29.5% and 41% of the total visits counted were in the neutral position and not taken into account in determining the differences in attractiveness of PVY infected and uninfected potato plants to aphids. This is in line with the vast majority of aphid behavioural studies (Ninkovic et al., 2011; Pettersson et al., 1994; Quiroz and Niemeyer, 1998). It could however be argued that not making a choice is also a decision and should be included in the analysis, which would probably lead to different results.

5.2) Early detection of PVY Infections via thermal imaging

Thermal imaging as tool for virus detection in potato

In this study it was shown that mid- infrared imaging is applicable to check potato plants for PVY infections. Correlations between the observations on thermal images and the ELISA tests were moderate. But when plants were separated into age groups, it became clear that the accuracy of this method is increasing with the age of the plants. From the second week after sprouting PVY infections could be determined with 69- 76% accuracy, depending on the cultivar, in the third week 90-95% were determined correctly. The findings are in line with studies on other species in which viral infections could be detected (Jinendra et al., 2010). Still it is not clear what actually causes the alteration of thermal signatures in PVY infected plants. It might be the change in leaf surface (wrinkly due to infection) which

reflects radiation differently or a physiological change like stomatal evapotranspiration which leads to a different heat signature.

In connection to the first part of the study it became clear, that the detection of PVY can be done before aphids can use secondary infections as a new virus source. The peak of aphid migration in Sweden is usually in July. At this time of the potato growing season, plants are between 20-25 cm high (Sigvald, 1985). At this growth stage a successful discrimination between healthy and infected plants on the basis of mid infrared images can be done with a high accuracy. So infected plants can be removed and inoculum source for PVY can be mitigated.

Possible improvement of the technique

The current study was a first evaluation of the possibilities of mid infrared remote sensing in virus detection in potatoes. It is therefore obvious, that a lot more research has to be conducted and the technique can be improved. As a next step, picture analysis with computer based algorithms has to be introduced, to produce more objective data. The applied method of visual determination of infected and healthy plants is not objective, as most of all, experience can have a big influence on the accuracy of correct determination. Furthermore, knowledge of the shape of infected leaves gives the possibility to judge by morphology of the plant and not only by thermal cues. The higher accuracy in the King Edward cultivar might be attributed to this. In this cultivar PVY infections have a high impact on the leaf shape from a very early stage of growth on. With this knowledge of morphological differences in King Edward it is easier to determine infected plants than in Solist.

Further it cannot be ruled out, that the plants were infected with other viruses as ELISA tests were only run for PVY. Also other stresses than the PVY infection might have caused the different patterns on thermal images (Chaerle and Van Der Straeten 2000). However, this is very unlikely, as in this study all plants were grown under the same conditions (soil, water, light etc.) and if other stresses were

responsible for the change in infrared images, all plants should exhibit the same patterns on infrared images.

Future outlook for thermal imaging in practise

The method of virus sensing has potential to be applied in practise. As PVY has a very wide host range, once established in potato it could be transferred into, for example, tomato production and many other agriculturally used *Solanacea* species. For seed potato production the technique could be extended to tubers directly. If it would be possible to reliably detect infected tubers with infrared, it would be possible to replace laborious and time-consuming serological tests and enable a high throughput checking of seed material. Also for breeding purposes this approach could be feasible. Plants which show no symptoms when infected with PVY can easily be mistaken as resistant. But this assumption is misleading and the invisible infection still has an influence on the yield (Hane and Hamm, 1999; Weidemann, 1988). With infrared, virus infections could be detected, even though no visible symptoms occur. However, the technique has weaknesses as well. The camera used in this study has a very sensitive focal depth and it is hard to obtain sharp pictures usable for discrimination of infected and healthy plants. Further the high price of cameras operating in mid infrared range is an obstacle. So in order to use this approach in practise a cheaper and easier to handle camera would be preferable and has to be looked for.

6. Conclusion

It became clear, that the factor time is crucial for both, the attraction of aphids via volatiles to PVY infected plants, as well as for the detection of infections with infrared methods. Our finding that viruses attract aphids in the early stage of growth, implies, that detection of viruses in plants must be done as early as possible. The proposed method by mid infrared sensing allows this and an early rouging of diseased plants in seed potato production, to prevent the spread of PVY.

7. Acknowledgments

I would like to thank my supervisors Velemir Ninkovic and Tobias Lindblom for their patience and time contributed to me.

As well as Linda Deißler and Helen Stewart for critical proofreading and help with the language.

8. Appendix

DAS- ELISA protocol

1. Dilute coating antibody in coating buffer as recommended on bottle label. Add 100 µl to the required number of well for your test, or fill the whole plate.
2. Wrap the plate in cling film. Incubate the plate at +37° C for 4 hours or incubate at +4°C over night. Can be stored for some weeks in +4° C.
3. Wash the plate three times with PBS+ tween 20. To do this fill the plate with PBST and invert to remove the buffer. Try to make the plate as dry as possible.
4. Press your leaf in the leaf juice press with sample buffer (extraction buffer). Collect the sap in small test tubes and put them in labeled boxes. Put box for store at -20° C.
5. Wash your coted plate as in (3) above and fill them with 100µl of your sample and with positive and negative control. Follow layout on you protocol. Wrap plate tightly with cling film and incubate at +4° C overnight (at least 16 hours)
6. Wash the plate as described in (3)
7. Dilute the conjugate in conjugate buffer as recommended on the bottle label. Add 100µl to each well with conjugate incubate 2 hours in +37°C.
8. Wash the plate as described in (3)
9. Prepare the substrate just before use. Dissolve phosphatase substrate 10 mg in 100ml of substrate buffer for each plate.
10. Add 100 µl substrate to each test well. Wait for one hour and read on ELISA reader at 405nm.
11. Positive results should be double negative control.

Buffers

Coating buffer: Dilute one coating buffer tablet in 100 ml distilled water pH 9,6

Wash buffer: Dilute one PBS tablet in 1000ml distilled water pH 7,4 and add 0,5 ml Tween20

Sample buffer (extraction): Add 20g Polyvinylpyrrolidone (PVP) in 1000ml PBS+Tween20

Conjugate buffer: Add 2g albumin in 1000 ml sample buffer.

Substrate buffer: 100ml Dietanolamin in 800ml distilled water. Adjust pH to 9,8 with HCL. Add distilled water up to 1000ml.

All buffers should be stored in refrigerator.

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Pictures and Diagrams:

Title picture “Aphid”:

Photography by Scott Bauer, distributed under Public domain.
<http://www.ars.usda.gov/is/graphics/photos/sep01/k9602-1.htm> (20.08.2016)

Figure 1:

Data from FAO Stat <http://faostat3.fao.org/browse/Q/QC/E> (20.08.2016)

Figure 3 a:

Photography by Chrisvis1, distributed under Creative Commons Attribution-Share Alike 3.0 Unported license. https://commons.wikimedia.org/wiki/File:Necrotic_ringspot.JPG (20.8.2016)

Figure 4:

Modified after: Edgar Schliephake

Figure 6:

Modified after: Dimitrije Markovic