Final report (Proposal K112146)

The most important results of the proposal will be summarized as follows:

1. **Methodological considerations and results**
2. **Characterization of the biological significance of putative signal compounds of acquired resistance in tobacco: azelaic acid (AzA), pipecolic acid (Pip) and N-hydroxypipecolic acid (NHP)**
3. **Role of spectral distribution of light in the induction of SI/SAR**
4. **Testing of other abiotic and biotic factors**
5. **Conclusion**

Systemic immunity (SI or systemic acquired resistance, SAR) is a defence mechanism that induces protection against a wide range of pathogens in distant, pathogen-free parts of plants after a primary inoculation. In the present case TMV-tobacco (*N. tabacum* Xanthi *nc*) plant-pathogen system was used in all studies for SI/SAR induction (Ross, 1961a; Manosalva et al., 2010). Multiple mobile signal compounds were identified as putative SAR signals or important factors for influencing movement of SAR signalling elements in Arabidopsis and tobacco. These include compounds with very different chemical structures like lipid transfer protein DIR1 (*D*efective in *I*nduced *R*esistance1), methyl salicylate (MeSA), dehydroabietinal (DA), azelaic acid (AzA), glycerol-3-phosphate dependent factor (G3P), the lysine catabolite non-protein amino acids, Pip and NHP (Fig. 1). SI/SAR signalling was one of the most debated questions in plant pathology in the last two decades (for review see Ádám et al., 2018). Local form of acquired resistance response was also described. In this case resistance could be manifested on the treated (local) and the opposite, induced half (distant effect) of the leaf after chemical treatment or infection of only one half of the same leaf surface (local acquired resistance, LAR) (Ross, 1961b). The distant effect with LAR could be more pronounced especially with putative signal compounds than during systemic induction. Therefore in some cases either SAR or LAR exper-

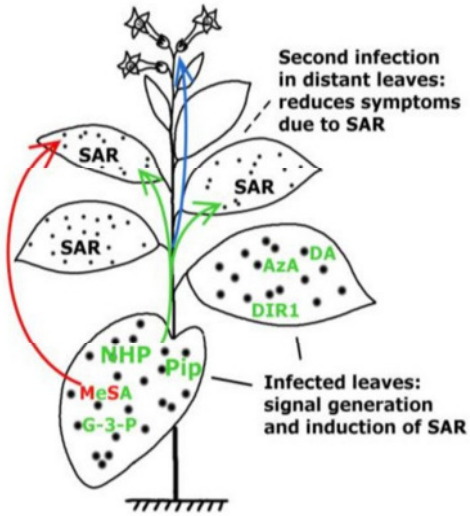


Fig. 1 Schematic view of the development of systemic immunity (SI, systemic acquired resistance, SAR) in plants. Main putative signal molecules: methyl salicylate (MeSA), lipid transfer protein DIR1 (Defective in Induced Resistance1), dehydroabietinal (DA), glycerol-3-phosphate (G3P) or G3P-dependent factor, azelaic acid (AzA), pipecolic acid (Pip) and its derivative, N-hydroxypipecolic acid (NHP) move from the imducing leaves (primary inoculation) to pathogen-free distant parts of the plant where they induce SI against broad range of biotrophic and hemibiotrophic pathogens (second infection). The arrows indicate the movement of signal molecules via phloem transport (green) or the air (red arrow for the putative volatile compounds of airborne signals). The blue arrow indicates transgenerational SI signalling where the epigenetic information is inherited and present in the next generation (from Ádám et al., 2018 with modifications, *Inernational J. Molecular Sciences 19*(4), 1146).

imental system was followed in tobacco.

1. Methodological considerations and results

Althought the most important aim of the project was the characterization of the chemical signal compounds of SI/SAR induction in tobacco, there were basic difficulties with data collection, evaluation, digitalization, statistical analysis of the symptoms of infected plants and timing of signal generation during SI/SAR induction. Therefore these problems were solved before other studies.

1.1. Data collection, digitalization, evaluation and statistical analysis of the symptoms of virus infected plants

In the former studies either number of viral necroses per leaf area or the mean diameter of individual viral lesions/necroses were used for the characterization of plant resistance response to viral necrotic infections. However this view faces different problems. First of all the methodological approach is very important factor in the correct evaluation of the effectiveness of a signal transduction component of SI/SAR induction. The standardisation of the amount of viruses and the effectiveness of infection due to manual inoculation is a difficult problem. Therefore rather the diameter of viral lesions was used in recent studies for the evaluation of resistance response based on limited number (20-50) of lesions collected by chance (Ádám és Nagy, 2016). For example Manosalva et al. (2010) applied digital caliper to measure the diameter of individual TMV lesions in tobacco plants.

According to our experience the selection of eye-visible necroses by chance considers unevenly necroses/lesions with different sizes: small and bigger necrotic spots are overrepresented (Nagy et al., 2016). Therefore in the present experiments the changes in the mean of lesion diameter was considered as a basis for the evaluation of resistance respose but all spots on a leaf/half leaf (in some cases intervenial areas) were considered. In addition spots unvisible to naked eyes were also taken into consideration after magnification (Figs. 2, 4 and 8). A new method was developed for the collection, digitalization, evaluation and statistical analysis of data (Nagy et al., 2016; Ádám és Nagy, 2016). Usually four days after TMV (strain U1) infection (in *NahG* plants after 3 days) the tobacco leaves (*Nicotiana tabacum* L. cv. Xanthi *nc NN*) were detached and scanned with a HP Scanjet G2k 710 scanner to obtain high-resolution (300-400 dpi) digital images. The ImageJ 1.48v image analysis software (Schneider et al. 2012) was used for lesion selection and lesion size calculations after threefold magnification (all together 20-30x magnification of the original size) of leaf images on a computer screen. However, due to the low contrast of small TMV spots, lesion selection was done manually using a drawing tablet. Lesion size was expressed as the mean of the major and minor axes of the best fitting ellipse having equal area to the lesion. All calculations were carried out with R (R Core Team 2015). The Shapiro–Wilk w test for normal distribution of lesion size was calculated using function 'shapiro.test' with its default settings. For comparison of sample means, a multiple comparison procedure was used with the R package multcomp (Hothorn et al. 2008). The method allows simultaneous comparisons, while the familywise error rate, used as the standard measure for false positive results in multiple testing, remains well controlled (Herberich et al. 2010). Furthermore, this method tolerates well heteroscedasticity of samples (unequal variances, non-normal distribution of data and unbalanced group sizes).

Further methodological aspects are outlined in Fig. 2 (Ádám and Nagy 2016). Virus (TMV) dilution did not influence the diameter of viral lesions considerably but decreased the number of lesions (by about 60-70% after 4x dilution of viral concentration in the inoculation media). The density of TMV lesions above a certain threshold level (3.0-3,5 lesions cm^{-2}), however formed interconnected groups. The lesion diameter of the lesions forming a group was significantly lower than the mean diameter of double and single lesions (by 22,2% and 18,9%, respectively). These results suggest that the value of

lesion diameter is not influeced by a wider range of dilution (1-4 times) and relatively independent of lesion density but above a certain threshold the formation of lesion groups can lead to underestimation of the results by about 10-15% (depending on its ratio in the total lesion population). Therefore grouped lesions should be omitted from further evaluation (Fig. 2, Ádám and Nagy, 2016, published in Hungarian).

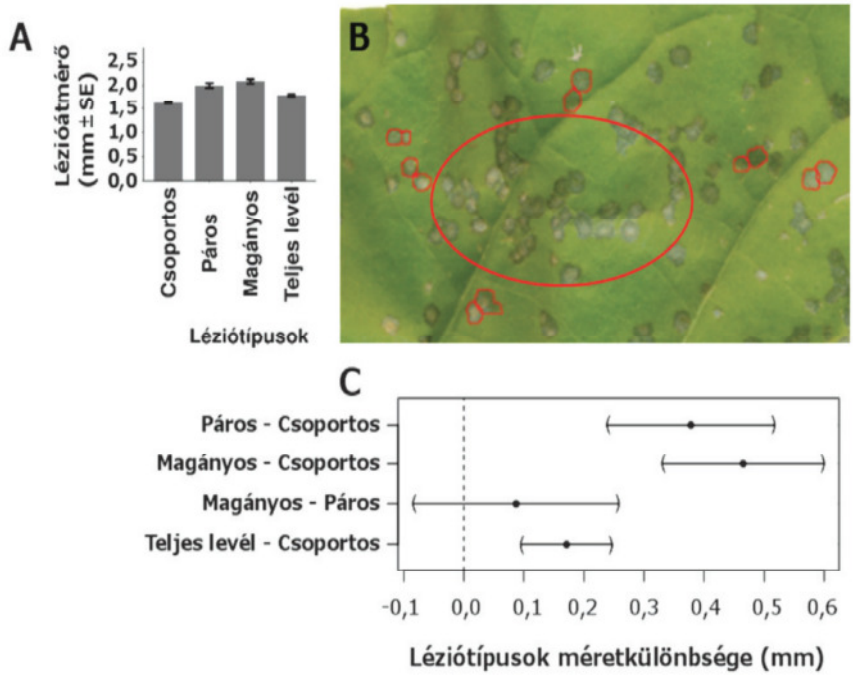


Fig. 2 Mean diameters (A) of different lesions (single, doubled and grouped as indicatewd by red ink in B) of tobacco mosaic virus (TMV) infection in Xanthi nc tobacco plants and multiple comparison of means (C). The difference is considered significant at 95%, if the confidence interval does not cross the vertical dashed line representing a difference of 0 in subfigure C. (From Ádám and Nagy, 2016, *Növényvédelem (Plant Protection)*, *77*(9), 430-461, published in Hungarian)

This complex approach and new method was used in several studies including the role of azelaic acid in signalling and induction of SI/SAR (Figs. 3,4,5 and 6, Nagy et al., 2016a) and the role of spectral distribution of light in resistance response against TMV infection (Figs. 9 and 10, Nagy et al., 2017b) The results with two other putative inducers or signal compouds of SI/SAR, pipecolic acid and N-hydroxypipecolic acid are also detailed (Figs. 7, 8 and Table 1) (publication is in prepartion).
This method could be applied after limited modifications to the evaluation of other necrotic viral and non-viral infections as well. On the other hand for example we applied successfully our method after tissue staining with diamino-benzidine (DAB) to evaluate H_20_2 accumulation in/around the lesions (data not shown).

1.2. The timing of SI/SAR induction in tobacco-TMV plant-pathogen system

The timing of the signal transduction events during SI/SAR development is crucial for the detection and characterization of the putative signal compounds in the present TMV-Xanthi *nc* tobacco system. Timing of signalling events could be different in different plant-pathogen systems during SI induction. Therefore a method was developed to study the timing of the formation and movement of the putative signal compound(s) from the lower, inducing leaves into distant (upper) leaves (Nagy et al., 2016; Ádám and Nagy, 2016).
This method is based on the sequential removal (2 or 4 days after primary inoculation) of the inducing four bottom-most leaves and the detection of the effect of this procedure on the resistance response of distant leaves after challenge (secondary) inoculation. Sequential removal of the lower,

inducing leaves 2 or 4 days after primary TMV inoculation indicated that signal transduction from inducing leaves into distant ones (i.e. formation and movement of the signal) is fully completed within 4 days but was not yet detectable after 2 days. Hovewer, for the manifestation of the signal in the distant leaves is required further 3-4 days, consequently the second (challenge) inoculation was performed 7-8 days after the primary inoculation. Thus, the phloem sap from the lower inducing leaves was collected in this time window (2 or 3 days after primary TMV inoculation) for chemical analysis in the subsequent experiments (Nagy et al., 2017a).

2. Characterization of the biological significance of putative signal compounds of acquired resistance in tobacco: azelaic acid (AzA), pipecolic acid (Pio) and N-hydroxypipecolic acid (NHP)

Former studies in the literature indicated that salicylic acid (SA) as a signal compound *per se* plays no role in the induction of SI (but its presence is required for the development of SI) in *Arabidopsis* and tobacco. On the other hand, methyl-salicylate (MeSA) also has a limited signalling function in Arabidopsis only under certain light (darkness after infection) conditions (for review see Ádám et al., 2018). More recent studies indicated that several hours (at least 6 h) of light exposition after primary inoculation with bacteria or TMV restore the SAR minus phenotype of *bsmt1* (*b*enzoic acid/salicylic acid *m*ethyl*t*ransferase1, responsible for MeSA synthesis from SA), *dir1-1* (putatively involved in the movement of the signal compound), and *sfd1*/*gly1-1* (*s*uppressor of *f*atty acid desaturase *d*eficiency1, responsible for the production of a glycerol-3-phosphate dependent factor) mutants in Arabidopsis and tobacco (Attaran et al. 2009; Liu et al. 2011a,b). In other words, the timing of the dark period relative to the primary inoculation severely influences the significance of a certain signal transduction compound in SI induction. However, under our experimental conditions tobacco plants were always exposed to light at least for 8-10 hours after viral or bacterial infection and treatments with SI/SAR signal compounds. If not, special conditions will be indicated. Considering these facts, three candidates of SI/SAR signalling were studied in tobacco-TMV plant-pathogen system: AzA, Pip and NHP (Fig. 1).

2.1. Azelaic acid (AzA) as a putative signal compound during SI/SAR induction in tobacco

Considering AzA as a signalling molecule in SAR induction in Arabidopsis, Jung et al. (2009) reported that although AzA accumulated at elevated levels (6–7 times) in phloem exudates during bacterial induced SAR, external application of AzA *per se* did not promote SA accumulation and had minimal effect on gene expression but could induce local and systemic resistance. However, AzA-treated plants produced elevated systemic induction of SA accumulation and signalling upon bacterial inoculation (*P. syringae* pv. *maculicola* strain *Pma*DG3) in distant leaves with enhanced resistance against the pathogen. Later studies suggested that AzA itself does not function as a systemic, long-distance signal molecule but rather locally induces SAR signal(s) emission in primary infected tissue via AZI1 (*A*zelaic *A*cid *I*nduced1) protein accumulation (Cecchini et al. 2015).
AzA treatments in our studies caused locally little (about 10% increase or decrease) or no effect on the mean of lesion diameter for both the third or fourth leaves. The low concentration treatment (0.2 mM AzA) decreased the lesion diameter (about 0,15 mm) compared to the control, but higher concentrations had no effect or even increased the lesion size. However, we noted that AzA concentrations higher than 2.0 mM had toxic effects (30–50% of the area of the infiltrated leaf was necrotized) (data not shown). Comparison of multiple sample means showed significant decrease only between control leaves and 0.2 mM AzA-treated leaves both for the third and fourth leaves (Fig. 3a). However, these differences showed only 8.5 and 7.6% decreases in lesion diameter, respectively, and their biological relevance is not clear (Nagy et al., 2017a, calculated from Experiment 1 in Supplementary Table 2). As a comparison, leaf position (from the third to sixth leaves) influenced lesion size of control plants by 32.8% in the same experiment.

Induction of SAR by TMV inoculation of the lower four leaves caused a significant decrease in lesion diameter of distant leaves following the challenge (second) TMV inoculation . This effect was more pronounced on leaf level 5 than on leaf level 6. On the contrary, AzA treatments did not induce significant systemic changes in lesion diameters either on the fifth or sixth leaf levels at any concentrations as compared to control plants (Fig. 3b). Moreover, comparison between leaves with SAR (as a positive control) and distant leaves of AzA-treated plants showed highly significant differences at all concentrations with the exception of 0.5 mM AzA treatment on leaf level 6 (Fig. 3b). These results also suggest that AzA plays no substantial role in SI/SAR induction. The same tendency of results was found in Experiments 2 and 3 (Nagy et al., 2017a, Supplementary Table 2). However, clearly, there was no concentration-dependent effect of AzA treatments neither locally nor systemically in any experiments. Jung et al. (2009) reported that AzA can induce either local or systemic resistance against bacterial multiplication over a wide concentration range from 0.1 to 1.0 mM in Arabidopsis in a concentration dependent manner. On the contrary, induction of SAR by TMV infection gave highly significant decrease of mean lesion size in all the three experiments (in most cases at p\0.001) (Nagy et al., 2017a, Supplementary Table 2, Figs. 3 and 4).

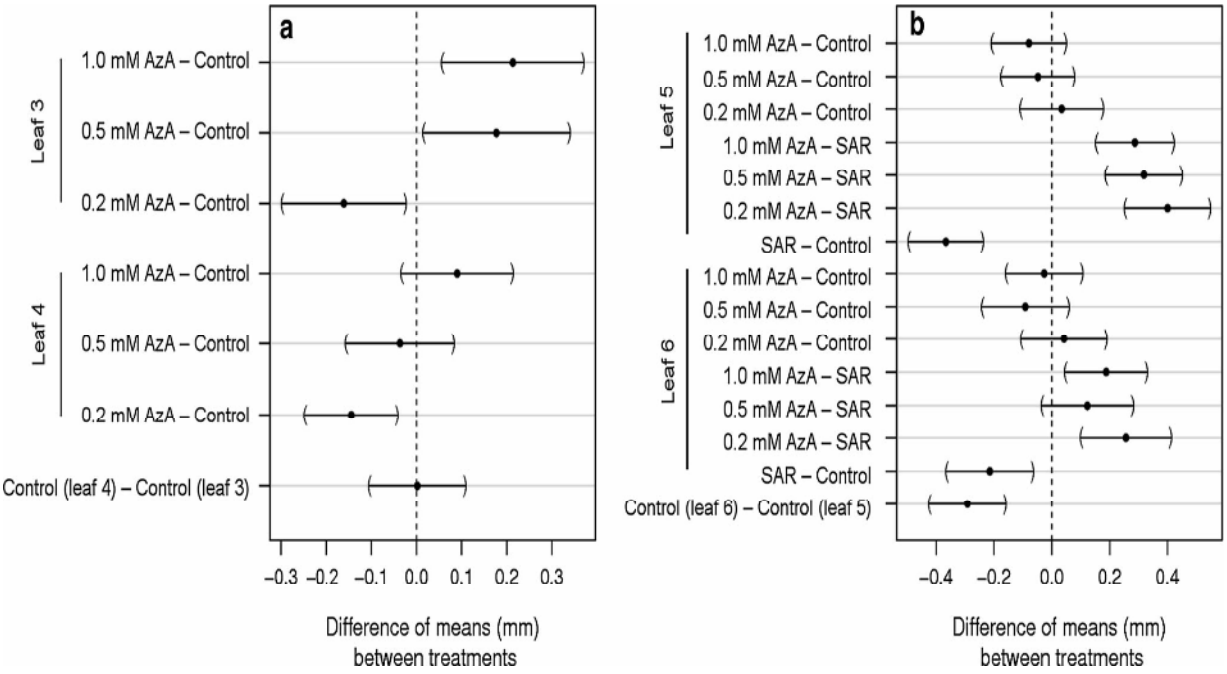


Fig. 3 Multiple comparisons between means of TMV lesion sizes after azelaic acid (AzA) pretreatments under „light" condition in tobacco. Local effects (a) were detected on leaves 3–4, whereas systemic effects (b) were detected on leaves 5–6. Each dot in the middle of the horizontal segments represents the difference between estimated means of the treatments. Brackets flank 95% confidence intervals. The difference of means is considered significant at 95%, if the confidence interval does not cross the vertical dashed line representing a difference of 0. Induction of SAR: plants were inoculated with TMV on the lower four leaves under light ciondition and challenged 7 days later on the fifth and sixth leaves without further treatments. Treatments were compared to their controls on the same leaf level (from Nagy et al. 2017a, *Acta Physiologiae Plantarum 39*:9).

As the former publications of the effect of AzA pretreatment on SI/SAR induction were performed in Arabidopsis bacterium plant-pathogen systems (Jung et al. 2009; Yu et al. 2013), we also analysed the effect of AzA on bacterium induced HR symptoms and multiplication of a compatible bacterium in tobacco plants. *P. syringae* pv. *tomato* (*Pst*, strain DC3000) is a well-known pathogen of Arabidopsis using a virulence factor, coronatine to attenuate SA-mediated defense mechanisms (Attaran et al. 2009). However, in a non-host plant, tobacco *Pst* causes quick hypersensitive reaction (HR) associated with cell death and induction of SA-dependent pathway (Liu et al. 2013). Therefore, besides *Pst*, we tested the effect of AzA on a compatible bacterium, *P. syringae* pv. *tabaci* in tobacco. *Pst* caused similar HR-like necrotic symptoms either in control or AzA pretreated local and systemic leaf panels 7–9 h after bacterial infiltration, in a bacterial concentration-dependent manner. Similar timing of HR induction of *Pst* was reported by Liu et al. (2013). The multiplication of *P. syringae* pv.

tabaci did not show considerable decrease after AzA treatment neither locally nor systemically. SAR-induced resistance response to *Pseudomonas* species often represents the inhibition of bacterial multiplication by several orders of magnitude (Attaran et al. 2009). Considering these results, AzA did not induce resistance response against bacterial pathogens in tobacco (Nagy et al., 2017a).

We have also performed experiments with tobacco plants kept in darkness subsequent to AzA pretreatment to test whether this condition can activate AzA-mediated local and/or systemic response in tobacco. The effect of local AzA treatment on TMV lesion size of local or systemic leaves after incubation in darkness did not show significant decrease as compared to control (but see the effect on SAR leaves induced by primary TMV infection) (Fig. 4).. Experiments with the multiplication of a compatible bacterium, *P. syringae* pv. *tabaci*, in local and systemic leaves after AzA treatment in darkness also showed no significant dicrease. These data clearly suggest that AzA-mediated signalling does not rely on factors activated in darkness, at least in tobacco plants (Nagy et al., 2017a).

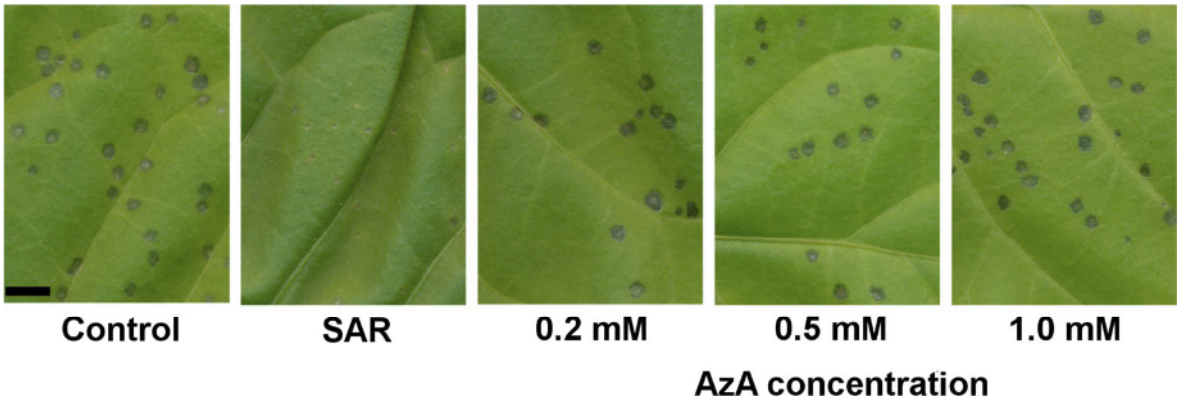


Fig. 4 Systemic effect of azelaic acid (AzA) pretreatment on TMV lesion size of *Nicotiana tabacum* cv. Xanthi nc plants. Control leaves were infiltrated with water. Plants were kept in the dark after AzA pretreatment for the rest of the daylight period and subsequent night. TMV inoculation was performed on the fifth–sixth leaves 7 days after AzA pretreatment. The sixth leaves are shown here. Photograps were taken 96 hs after TMV inoculation. Bar 5 mm (From Nagy et al., 2017a, *Acta Physiologiae Plantarum 39:9.)*

To analyse more AzA-mediated SI/SAR induction capacity of TMV-infected leaves, phloem sap was collected in the above indicated time window (2 or 3 days after inoculation) for 24 h from TMV-infected and control leaves (Nagy et al., 2017a). Interestingly, HPLC–MS assays detected, besides C9 AzA (1,9-nonadienoic acid), low amounts of two other dicarboxylic acids, suberic acid (1,8-octadienoic acid), and sebacic acid (1,10-decadienoic acid) in petroleum ether extracted petiolar exudates of both TMV-infected and control leaves 3 days after TMV inoculation (Fig. 5). AzA content was about double (220.0* ± 32.1 lg mL–1) in concentrated exudates of TMV-infected leaves as compared to that in control exudates (120.1 ± 12.3 lg mL–1, *p\0.05, t test, n = 3). But as compared to Jung et al (2009) the increase in Aza content was limited and concentrated (extracted in petroleum ether) exudates collected from TMV-infected leaves containing AzA-fraction did not induce a local resistance response after infiltration into tobacco leaves in our experiments. The amounts of suberic and sebacic acids were also increased in exudates of TMV-infected leaves. According to Jung et al. (2009), these two acids had no biological activity in SAR induction. Guelette et al. (2012) found several monocarboxylic acids (C_9 nonanoic acid and C_{12} dodecanoic acid) in exudates of healthy Arabidopsis leaves based on GC–MS analysis, but their biological significance is not clear.

In addition, studies on the level of virus multiplication, the expression of coat protein gene of TMV after AzA pretreatments did not show significant differences (Fig. 6).

Taken together, similar to our present results, Zoeller et al. (2012) also found that in spite of the bacterially inducible AzA accumulation in infected leaves, external local AzA treatment does not inhibit the growth of *Pst* (strain DC3000) in Arabidopsis. In line with these results, Vicente et al. (2012) reported that AzA pretreatment caused a barely detectable inhibition of symptoms and growth of *Pst* DC3000 bacteria in both treated and distant leaves of Arabidiopsis. Consequently our

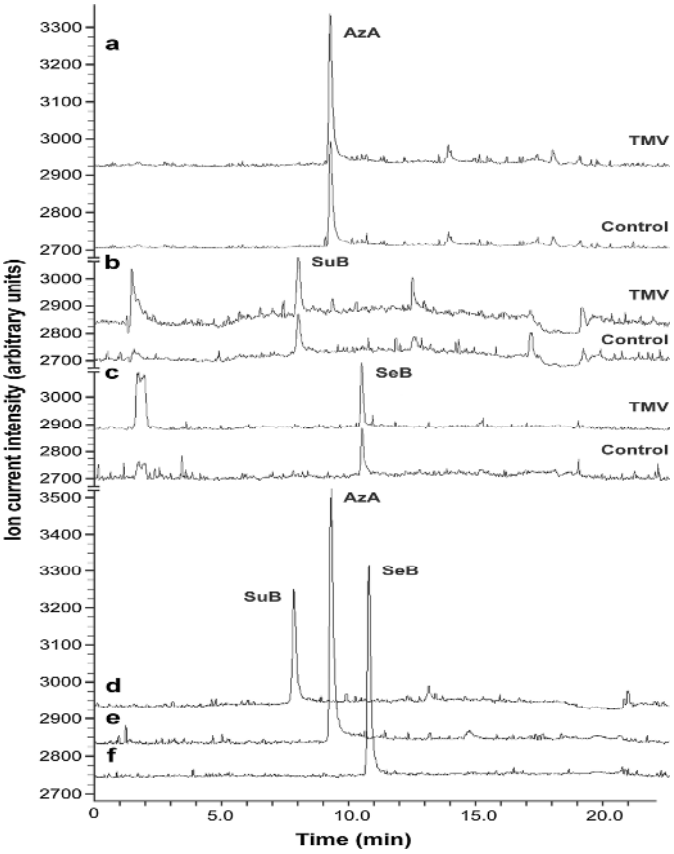


Fig. 5 HPLC–MS analysis of azelaic acid (AzA), suberic acid (SuB), and sebacic acid (SeB) (a–c) in phloem exudates of TMV-inoculated and control tobacco leaves, 3 days (72 to 96 h) after virus inoculation. Chromatograms of corresponding standards, AzA (m/z = 187), SuB (m/z = 173) and SeB (m/z = 201) are depicted in (d–f), respectively (from Nagy et al., 2017a, *Acta Physiologiae Plantarum 39*:9.)

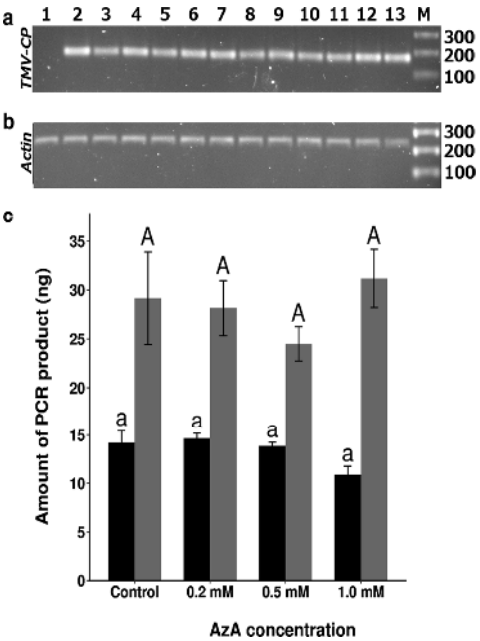


Fig. 6 Effects of azelaic acid (AzA) on transcript abundance of the TMV coat protein gene (TMV-CP) in TMV-inoculated tobacco leaves 3 days after virus inoculation as detected by semiquantitative RT-PCR (a). The expression of Nicotiana tabacum gene actin-9 (Act-9) (b) was measured as constitutive control. Lane 1 uninfected tobacco, lanes 2–13 TMV-inoculated leaves. Pretreatments: lanes 2–4 water infiltration, lanes 5–7 0.2 mM AzA, lanes 8–10 0.5 mM AzA, and lanes 11–13 1.0 mM AzA. M molecular weight ladder (bp) (Thermo Fisher Inc., Waltham, MA, USA). (c) Densitometric measurement (mean ± SE) of PCR products of TMV-CP gene (grey bars) and actin-9 gene (black bars). Different letters symbolise statistically significant differences (p = 0.05) after Kruskal–Wallis test applied separately to TMV-CP and actin gene products. Treatments were compared to their respective controls (from Nagy et al., 2017a, *Acta Physiologiae Plantarum* 39:9)

present results in tobacco and other studies in Arabidopsis suggest that in spite of the pathogen inducible lipid peroxidation mediated local accumulation in leaves and phloem exudates, AzA could not cause the signalling of SI/SAR and the induction of local and systemic resistance response against selected viral and bacterial pathogens (Nagy et al., 2017a, Ádám et al., 2018).

2.2. Pipecolic acid (Pip) and N-hydroxypipecolic acid (NHP): only NHP has a massive local and distant effect on the development of necrotic symptoms of TMV infection in tobacco

Similar to some other mutants with SI/SAR minus phenotype, Pip deficient mutant was described first without biochemical function in Arabidopsis after chemical mutagenesis. Although former genetic studies with an *ald1* mutant indicated the key role of an aminotransferase, ALD1 (*A*GD2-*l*ike *d*efense response protein 1) in local and systemic defence responses (Song et al., 2004), the function of Pip was discovered only later on (Návarová et al., 2012). In fact, detailed studies indicated that (i) ALD1 gene product shows *in vitro* substrate preference to lysine, a putative precursor of Pip biosynthesis in plants and animals (Hartmann et al., 2017); (ii) the biosynthesis of Pip in Arabidopsis is dependent on functional ALD1 locus (Návarová et al., 2012) and (iii) ALD1 enzyme acts as a first step during lysine catabolism and directly transfers the α-amino group of L-lysine to an oxoacid, preferentially pyruvate to form ε-amino-α-ketocaproic acid (KAC) and alanine. Next steps from KAC (cyclization, isomerization) are leading to the formation of Pip (Hartmann et al., 2017). Furthermore, ALD1 transcript accumulates in the pathogen-inoculated and distant pathogen-free leaves. The local and systemic immune defects of *ald1* mutant Arabidopsis after bacterial inoculation could be rescued by external application of Pip. From the point of view of signal transduction during SAR response, it is important to note that Návarová et al. (2012) found strong Pip accumulation in petiolar exudate of SAR-inducing *P. syringae* infected Arabidopsis leaves. However, whether Pip has a direct role in long-distance SAR signalling remains to be elucidated in the future. In two recent publications, a new SAR signalling compound, a FMO1 (*flavin m*onoo*x*ygenase1) activity dependent N-oxygenation product of Pip, N-hydroxypipecolic acid (NHP) was described in bacterial Arabidopsis systems (Hartmann et al., 2018; Chen et al., 2018). Wang et al. (2018) reported induction of AOS formation and dose dependent (0.1-2 mM) effect of Pip in SI induction of Arabidopsis (NHP was not studied at all in this paper). However, the role of NHP and Pip has not yet been compared strictly and studied in virus induced SAR/LAR mechanisms in tobacco (Ádám et al. 2018, 2019).

We detected earlier drastic local accumulation of two amino acids, Pip and tryptophan after two necrotic viral infections, TMV and cucumber mosaic virus (CMV, necrotic strain) infections in tobacco by HPLC-MS (Ádám et al., 2018). Treatment with Pip showed limited but significant activity against the symptoms of TMV-infection under two hydroponic experimental systems (Ádám et al., 2019).

Subsequent experiments, however faced difficulties. First of all, the N-hydroxylation product of pipecolic acid, NHP was not commercially available and separation of these two compounds from each other had no backgroud in the HPLC-MS literature (formerly NHP was analysed by GC-MS methods). Therefore the proposal was extended by one year. After several trials both problems was solved. NHP was synthesized from piperidine by a chemical company (Organofil Ltd., Budapest) in Hungary and its structure was justified by different spectroscopic methods. Separation and measurement of Pip and NHP was possible after derivatization by a fuorescent probe, fluorenymethyloxycarbonyl chloride (Fmoc-Cl) under reversed phase HPLC conditions used for protein amino acid analysis (Ziegler and Abel, 2014). Fmoc interacts either with primary (most of the amino acids) or secondary amines (for example proline, Pip and NHP). The structure of the fluorescent Fmoc derivatized products of proline, Pip and NHP standards and plant samples after HPLC separation (based on the fluorescent peak) was checked by HPLC-MS on the basis of corresponding m/z values and comparison to its correspondig Fmoc proline standard (Sigma). Measurements are in progress now to analyze the role of hydroxylation of Pip and the formation of

NHP during SI/SAR induction in tobacco. However, the biological significance of PIP and NHP against TMV infection was tested and compared in a local acquired resistance (LAR) test (see above).

To the contrary of AzA, Pip and NHP showed significant local and distant effects in tobacco (Fig. 7, unpublished results). But as it is clearly indicated in Fig. 7a and c, the effects of NHP and Pip were also different. First of all, NHP caused massive and significant dicrease (by about 65-70%, Fig. 8) in TMV lesion size within the whole concentration range (0,1-4,0 mM) (Fig 7a). Pip was active to a lesser extent only above 0,5 mM concentration (Fig 7c 28-32% decrease in lesion size). The effect of NHP showed no clear concentration dependence in this range (in other studies in Arabidopsis usually only one concentration of NHP was used). Moreover, NHP not only diminished lesion size, but caused differences in the types of TMV lesions (see T1 type lesions, where the small spots of 0,3-0,5 mm diameter lesions remained green showing superficially deeped lesion edges) summarized in Table 1. Distant effects on the opposite leaf halves are depicted in Fig 7b and d. NHP and Pip also induced significant resistance response in these leaf halves but at lower concentrations the effect was less significant. Preliminary HPLC-MS data suggested that Pip is not converted to NHP after infiltration in the absence of TMV infection but moved from the treated part into the opposite half of the leaves. This conclusion is also supported by the distribution of lesion types in different treatments: only NHP treatment caused the development of type 1 and 2 viral lesions either in treated or induced leaf halves (Fig. 8 and Table 1, unpublished results). Taken together the effect of Pip was limited as compared to NHP. Other important results suggest that the effects of NHP and Pip in transgenic *Nah-G* tobacco plants (salicylic acid is decomposed after formation in these plants) were not manifested neither in treated half leaves nor in induced half leaves (data not shown, unpublished results).

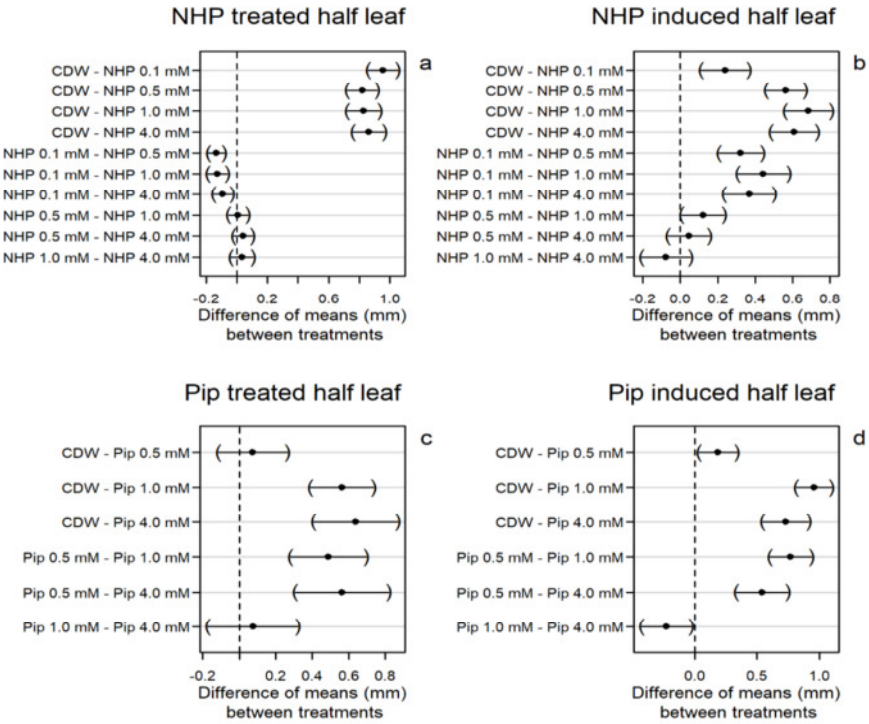
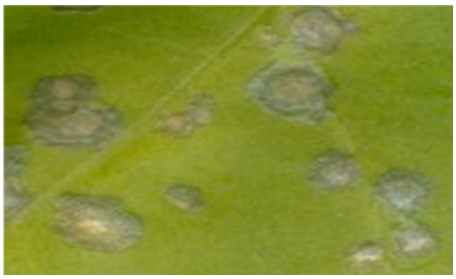


Fig. 7. Summary of multiple comparisons between means of TMV lesion sizes after N-hydroxypipecolic acid (NHP) (a,b) and pipecolic acid (Pip) (c,d) pretreatments in treated halves (a,c) and opposite, induced halves (b,d) of the same leaves. Local effects (a-c) were detected on half leves treated (infiltrated) with different cocentrations of Pip and NHP, whereas distant effects (b-d) were analyzed on the opposite half of the same leaf. Treatments were compared to their correponding controls and to each others. The difference of means is considered significant at 95%, if the confidence interval does not cross the vertical dashed line representing a difference of 0 (unpublished results)

These facts indicate that NHP acts against TMV on salicylic acid (SA-) dependent manner. Moreover, SA was active against TMV-infection only in treated half leaves (T2/T3-type necroses became smaller)

but did not show distant effect. Consequently, NHP can act on SA-dependent manner, but NHP could be also responsible for the distant effect and drastic modification of lesion size and type (from T3 to T1/T2 lesions). Surprisingly, our preliminary data showed that neither NHP nor Pip can decrease the multiplication of TMV based on the expression of TMV coat protein gene. We studied PR1 expression as well. Although PR1 protein has only antifungal activity, its expression is considered as a marker of SI/SAR induction. NHP treatment caused more pronounced increase in PR1 expression than Pip treatment suggesting also differences in the functional roles of these two molecules. These results will be published partly at the National Plant Protection Days (February, 2020) and in the special issue (Mechanisms of Light Stress and Light-Related Acclimation Processes) of the International Journal of Molecular Sciences (IF:



Control (M.: 20x)

0,5 mM NHP (M.: 20x)

Fig. 8. Massive effect of N-hydroxypipecolic acid (NHP) infiltration on lesion size and type in treated half leaf (T1 and T2 type lesions) as compared to the control infiltrated with distilled water (T3 type usual lesions) (unpublished results)

Plant genotype/Treatment	**THL**	**IHL**
Xanthi *nc* NHP 0,1 mM	T1	T2/T3
Xanthi *nc* NHP 0,5 mM	T1/T2	T1/T2/(T3)
Xanthi *nc* NHP 1,0 mM	T1/T2	T1/T2 (T3)
Xanthi *nc* NHP 4,0 mM	T1/T2	T1/T2 (T3)
Xanthi *nc* Pip 0,5, 1,0 and 4 ,0 mM	T3	T3
Xanthi *nc* TMV (LAR induction)	T3	T2
Xanthi *nc* Control (DW)	T3	T3
Xanthi *nc* SA 0,5 mM	T2/(T3)	T3
NahG NHP (0,1-4,0 mM)	T3	T3
NahG Pip (0,5-4,0 mM)	T3	T3
NahG TMV (LAR induction)	T3	T3
NahG-Control (DW)	T3	T3

Table 1. Types of TMV necroses/lesions in treated half leaves (THL, local effect) and induced half leaves (IHL, distant effect). THL was treated with different compounds or TMV (in the case of viral induction of local acquired resistance, LAR) with the concentration indicated above in different genotypes (*NahG* and *Xanthi* nc *NN*) of tobacco (*N. tabacum*). Five days later both leaf halfves were infected with TMV. Salicylic acid (SA), NHP and Pip treatments required only 48 hs incubation for the full development of local and distant resistance response, but viral induction needed 4-5 days for distant response. Therefore in all treatments/experiments a 5 days interval was used for the development of local and distant responses. Types (T) of TMV lesions/necroses: T1 (tiny green deeped spots), T2 (whitish small spots) and T3 (large lesions surrounded by darkish ring, usually known as TMV lesions in Xanthi *nc* tobacco). DW, distilled water. Syllables are in brackets if proportion of a given lesion phenotype was limited (unpublished results)

4,183). Light dependence of TMV and NHP-inducible resistance will be also studied.

3. Role of spectral distribution of light in the induction of SI/SAR

In addition to former results in the literature on the positive effect of light exposition on SI/SAR induction (Attaran et al., 2009; Liu et al., 2011a) after primary inculation, we studied the effct of spectral distribution of light on TMV resistance and SAR induction in tobacco (Nagy et al., 2017b). This point is especially interesting as most experiments are performed under artificial light

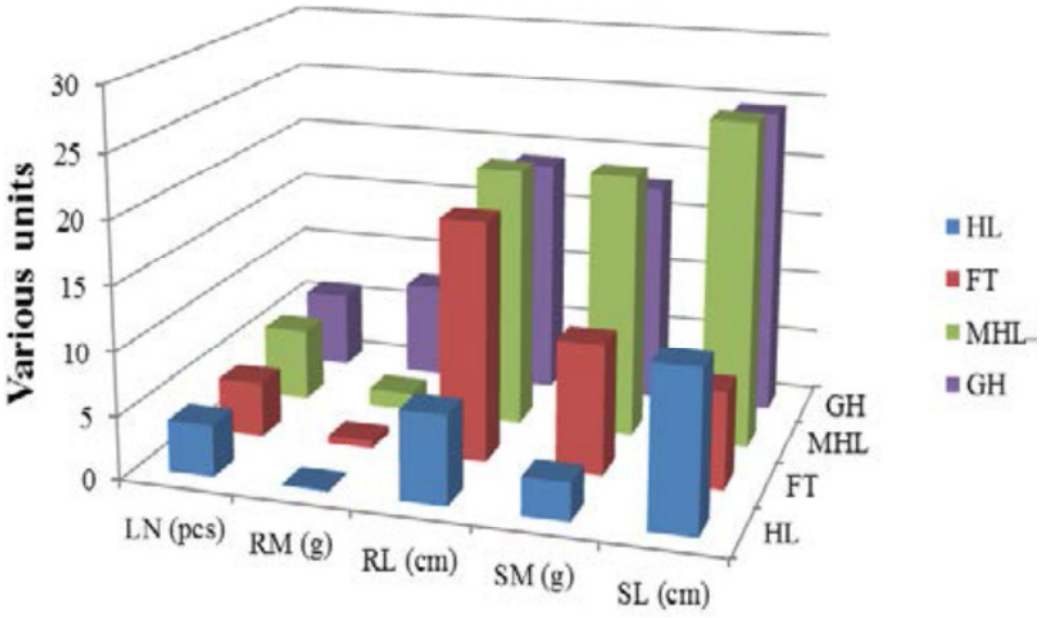


Fig. 9 Effect of spectral distribution of different artificial light sources on growth and development of tobacco (*Nicotiana tabacum* cv. 'Xanthi' *nc*). Plants were grown under different light sources, fluorescent tubes (FT), halogen lamp (HL), metal halide lamp (MHL) and greenhouse (GH) conditions during their entire lifetime. Statistical analysis is given in Supplementary Table 1. LN: leaf number (pcs, pieces); RM: root mass (g); RL: root length (cm); SM: shoot mass (g); SL, shoot length (from Nagy et al. 2017b, *Notulae Botanicea Horti Agrobotanici 45*(1): 270-275)

conditions. Different artificial illumination conditions caused drastic effects on growth and developmental parameters of plants, especially on root growth and mass, shoot mass and shoot lenght (Fig. 9). For example fluorescent tube (FT) light resulted in serious stunting effect, dark green leaves with shortened internodes and reduced number of leaves (Fig. 9). Halogen lamp (HL) light caused retarded growth, smaller root and shoot mass, high SM/RM ratio, long internodes and decreased number of leaves. HL and FT light showed very different spectral distribution, abundance or shortage in red and far red light, respectively. Among spectral distributions of light sources used, MHL (metal halide lamp) was the most similar to sunshine in the greenhouse (Nagy et al., 2017b, Supplementary Fig. 1).

The effect of different light conditions with very different spectral distribution was also manifested in the resistance to TMV infection especially if plants were exposed to different light conditions during their entire life time (Fig. 10a and c) not only *after* TMV infection (b and d) as indicated by TMV lesion size distribution (density) curves (a and b) and multiple comparison test for 95% confidence intervals (Fig. 10c and d).

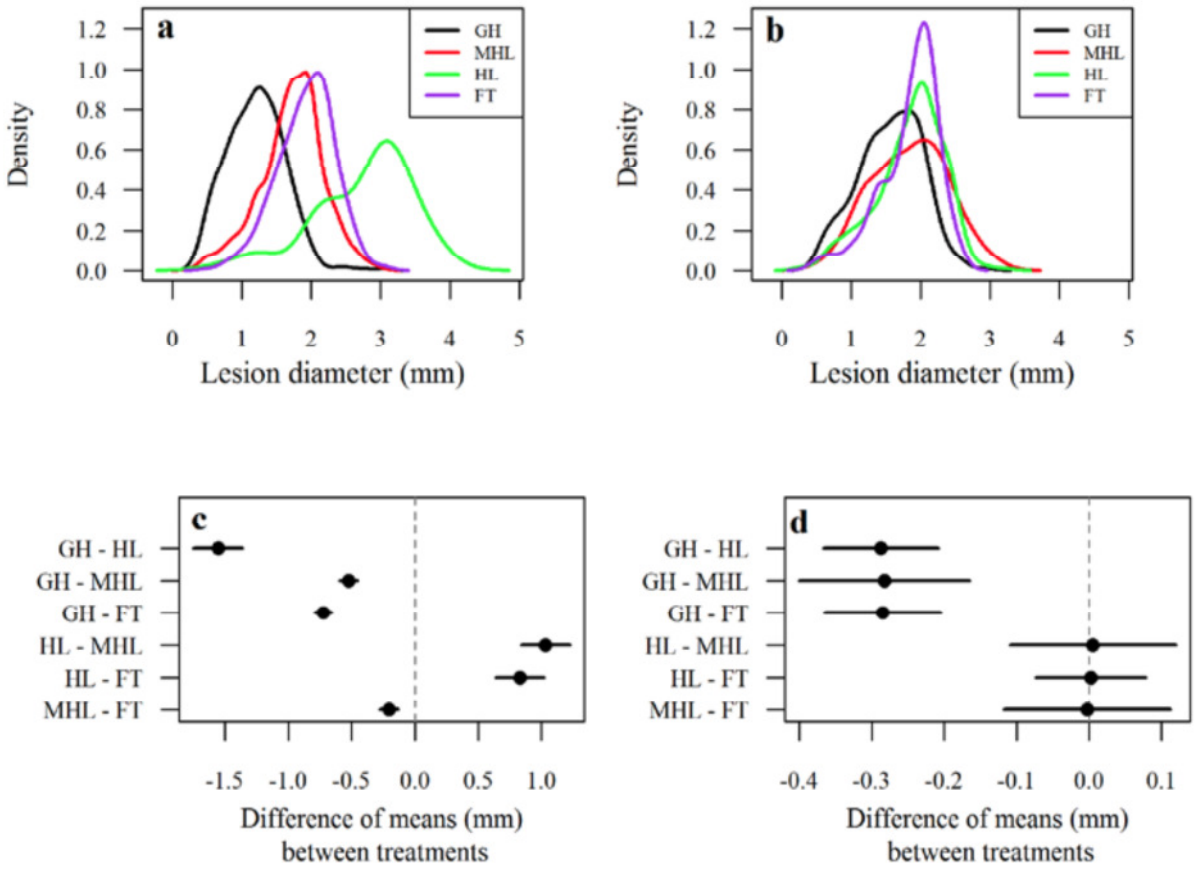


Fig. 10 Effects of different artificial light sources and greenhouse conditions on local resistance response of tobacco plants (*Nicotiana tabacum* cv. 'Xanthi' *nc*) to TMV infection. Kernel density estimation of TMV lesion size distribution (a,b) and multiple comparison of means (c,d) on leaf level 5 of plants kept under various light sources for their entire lifetime (a,c) or moved from greenhouse to different light sources only after TMV inoculation (b,d). Multiple comparisons of mean TMV lesion size were also computed on leaf 5. Dots represent the difference of the estimated means between treatments. Horizontal segments flank the 95% confidence intervals. The difference is considered significant if the confidence interval does not contain the 0, represented by a vertical dashed line. GH: greenhouse; MHL: metal halide lamp; HL: halogen lamp; FT: fluorescent tube (from Nagy et al., 2017b, *Notulae Botanicea Horti Agrobotanici*, *45*(1): 270-275)

As compared to greenhouse (GH) conditions, light sources with different spectral distribution influenced not only local resistance response but SAR induction capacity of plants as well (Nagy et al., 2017b, Fig 4a and b). After induction of SAR, the effect size (proportion of mean lesion diameter of SAR induced and corresponding control plants) was evaluated in two different leaf level under different light sources. MHL and GH conditions induced higher degree of SAR response on fifth and sixth leaves (about 40-50% reduction in lesion size, (Nagy et al., 2017b, Supplementary Table 2). As it was predictable from the spectral analysis, GH and MH lights were relatively close to each other and so was their effect on SAR induction capacity (Nagy et al., 2017b). In addition, HL and FT lights had deficiencies in their spectra compared to natural light (GH), and their effects were also reduced (i.e. manifestation of SAR decreased). Thus, bootstrap confidence intervals in Fig. 4a and b (Nagy et al., 2017b) clearly indicate that GH conditions and MH light source significantly differ from FT and HL light sources. Despite the fact that under GH conditions light-dependent factors (for example seasonal fluctuation in photoperiod) and other environmental factors than light also can influence SAR induction, the results presented and the spectral similarity of MHL to sunshine (Nagy et al., 2017b, Supplementary Fig. 1) suggest a specific spectral distribution of light that promotes SAR induction.

4. Testing of other abiotic and biotic factors

Two prooxidant compounds (hydrogen peroxide and sodium chlorate) with different concentrations were tested in chemical (abiotic) SI/SAR induction. One of these, sodium chlorate ($KClO_3$) was reported to induce SAR against TMV in tobacco (Strobel and Kuc, 1995). However we could not induce neither local (sometimes the treatment damaged the leaves) nor systemic effects against TMV infection (unpublished, data not shown).

Preliminary studies were also performed on the transgenerational effect of SAR (Luna et al., 2012). After induction of SAR by TMV infection, seeds from individual plants were collected. (The development of SAR was checked separately in each plant by transient chellange inoculation on one of the upper leaves. This leaf was removed 4 days after challange inoculation.) Interestingly, plants with SAR showed significantly earlier flowering (10-12 days earlier than the control plants, 50,5 days after SAR induction). However, testing of F1 generation for SAR showed no increased resistance response in any of the F1 plants (8 replicates) as compared to control F1 plants (exposed to the same pocedure without SAR induction).(Unpublished results.)

Two more scientific papers on plant resistance mechanisms were published partly under the project (not connected directly to SI/SAR induction) by Juhász et al. (2015) and Albert et al. (2017) and listed under References.

5. Conclusion

A) A semi-automated new method was developed for data collection, digitalization, evaluation and statistical analysis of the symptoms of virus (TMV) infected plants based on ImageJ 1.48v image analysis software (Schneider et al. 2012) and R-surface (R Core Team 2015). This method was applied succesfully in different studies (Nagy et al., 2016; Ádám és Nagy, 2016; Nagy et al., 2017a,b; Ádám et al., 2018, 2019). This method after modifications could be applied for the evaluation of other necrotic viral and non-viral infections as well. On the other hand this method was used successfully after tissue staining with diamino-benzidine (DAB) to evaluate H_20_2 accumulation in/around TMV lesions (unpublished, data not shown).

B) The biological significance and mode of action of three putative signal transduction compounds of SI/SAR induction, azelaic acid (AzA), pipecolic acid (Pip) and N-hydroxypipecolic acid (NHP) were studied and compared in tobacco-TMV plant-pathogen interaction. SI/SAR signalling was one of the most debated issues in plant pathology in the last two decades. Our present results in tobacco and other studies in Arabidopsis suggest that in spite of the pathogen inducible lipid peroxidation mediated accumulation in infected leaves and phloem exudates, AzA could not cause the signalling of SI/SAR and the induction of local and systemic resistance response against selected viral and bacterial pathogens (Nagy et al., 2017a; Ádám et al., 2018). To the contrary of AzA, significant results were found with NHP treatment in tobacco-TMV system. Local accumulation of Pip was detected in two necrotic virus infections in tobacco (Ádám et al., 2018). NHP decreased much more effectively symptom expression (massive local and distant effects on TMV lesion size and type) than Pip and these effects were developed on SA-dependent manner. On the other hand SA treatment has no distant effect and locally was less effective on lesion size. The interplay of NHP and SA supports the former results on the requirement of SA in SI/SAR induction although SA *per se* was excluded as a signal compound. Present evidence rather suggests that NHP can act as an important and very effective compound in local and distant resistance response but NHP and SA may have different functional roles. NHP could function as a signal compound during SI/SAR/LAR induction. However, further studies (especially the role of the conversion of Pip to NHP in connection with signal function and testing of other non-viral pathogens) are required for its full justification and elaboration of the way of its putative practical application in horticulture and field crops (Ádám et al., 2018, 2019).

C) Besides intensity of light and the timing of light exposition after primary inoculation, the third light factor that can cause differences in resistance to TMV infection is the spectral distribution of light. Our results clearly indicated that spectral distribution of light sources influences (i) plant growth and development; (ii) local resistance response to TMV infection and (iii) SAR inducing capacity of tobacco plants. Certain light sources with unbalanced light spectrum had negative impact on plant growth and development, local resistance response and SI/SAR induction capacity of tobacco plants. Halogen lamp (HL) and fluorescent tube (FT) light sources showed very different spectral distribution, relative abundance or shortage in red/far red light, respectively. The more similar was the spectrum of the artificial light source to sunshine (greenhouse conditions), the stronger was the inducible SAR response. From a practical point of view, under artificial conditions, metal halide lamp or a mixture of HL and FT light sources can be suggested as optimal test conditions. Consequently, the optimization of the effect of artificial light sources is an important factor in experimental design studying signal transduction and biochemistry of SI/SAR (Nagy et al., 2017b).

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