Research Article

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Simple modifications of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acids toward new weapons against plant diseases

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Abstract: Recently, the biggest challenge in agriculture is the search for new, effective, and ecological methods of protecting plants against diseases. One of the fastest-growing and prospective strategies is a method based on activating the plant's natural defenses. The use of suitable substances (elicitors) stimulates the immune system of plants, which makes them resistant to infections even before the first symptoms appear. This article presents preparation, characterization, phytotoxicity, and plant resistance induction efficacy of 28 ester derivatives of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acids as potential inducers of plants' natural immune system. Plant resistance induction efficacy tests were performed on tobacco *Nicotiana tabacum* var. *Xanthi* infected by the tobacco mosaic virus (TMV).

Keywords: plant protection, systemic-acquired resistance, esters, phytotoxicity, tobacco mosaic virus

† Biological tests.

Synthesis.

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1 Introduction

Continuous population growth is a factor causing an increase in the demand for food, which forces agriculture to use more effective plant cultivation methods. Pathogens, insects, other pests, or weather conditions are serious threats that reduce crop yields. Currently, every year, up to 40% of global crops are destroyed by organisms harmful to plants [1]. Plant diseases and pests cause huge economic and environmental losses, which is adversely affecting people's standard of living. "Protecting plants, protecting life" – is the official motto of International Year of Plant Health 2020. One purpose of the IYPH 2020 initiative is to raise awareness about plant health in the context of protecting sustainable agriculture that will lead to increased food security at the global level by limiting the spread of harmful organisms to plant [2]. In the field of plant health protection, prophylaxis is much more effective and profitable than combating the consequences of pathogen infections.

One of the most promising ways to protect plants against pathogens is systemic acquired resistance (SAR) phenomena [3] – a natural plant defense mechanism that has been developed by plants through the evolutionary process. SAR has a broad spectrum action against pathogens [4,5]. This occurrence is initiated after pathogen attack or artificially by compounds imitating the plant–pathogen interaction (elicitors). Two groups of elicitors can be distinguished: natural (endogenous) like salicylic acid, azelaic acid, pipecolic acid; and synthetic – saccharine, 2,6-dichloroisonicotinic acid (INA), acibenzolar *S*-methyl ester (benzothiadiazole *S*-methyl ester, BTH) and their derivatives [6–13]. External application of SAR inducers (e.g., by spraying) activates the natural defense mechanisms of plants and, as a result, increases their disease resistance. This approach could be potentially used to develop new plant protection strategies [14].

Nicotinic acid (1), also known as niacin or vitamin B3, was first described by Hugo Weidel in 1873 [15]. It is

worth noticing that vitamin B3 is a water-soluble vitamin used by the body to form the nicotinamide coenzyme, NAD⁺, which functions in oxidation–reduction reactions and non-redox reactions [16]. Isonicotinic acid (**2**) is a derivative of pyridine with the carboxylic acid substituent at the 4-position. One of the most important derivatives of isonicotinic acid is isonicotinic hydrazine, which exhibits antimicrobial properties [17].

2,6-Dichloroisonicotinic acid (INA, **3**) was first discovered as a synthetic inducer of plant resistance against bacteria and fungi [18,19]. As shown in one of the first reports, the application of INA (**3**) in the field conditions increased the resistance of pepper, pear, and rice to diseases caused by pathogens [20]. Other studies show that 2,6-dichloroisonicotinic acid (**3**) stimulates SAR in tobacco plants, making it resistant to such pathogens as tobacco mosaic virus (TMV), *Cercospora nicotianae*, *Peronospora tabacina*, *Phytophthora parasitica* var. *Nicotianae*, and *P. syringae* pv. *Tabaci* [21,22]. INA (**3**) has been tested on many plant species in which it raised resistance against a broad spectrum of pathogens [23–27].

In our previous work, we have reported that the application of nicotinic acid (**1**) to tobacco plants increased its resistance to pathogens [28]. Moreover, isonicotinic acid (**2**) when derivatized into the salt form became biologically active, which resulted in a 45% reduction of necrotic spots caused by viral infection. In our latest work, we have reported that amide derivatives of isonicotinic acid and 2,6-dichloroisonicotinic acid were more biologically active than acids and their plant resistance induction properties were up to 92% [29]. As nicotinic (**1**), isonicotinic (**2**), and 2,6-dichloroisonicotinic (**3**) acids and amide derivatives showed promising results in stimulating plant immunity, we decided to take a closer look at the ester derivatives. The main aim of this article is to investigate new ester derivatives of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic and to find the correlation between the change in the chemical structure of the ester functional group of the elicitor and its impact on biological efficiency in inducing plant resistance against pathogens.

2 Materials and methods

2.1 Synthesis

2.1.1 Materials, method of synthesis, and spectra data

All commercially available reagents were purchased from commercial suppliers (Sigma Aldrich, Merck). Synthetic

protocols and all spectra data of obtained compounds are reported in the Supplementary Information.

2.2 Biological properties evaluation

2.2.1 Phytotoxicity assessment

Phytotoxicity tests were performed on two plant models: (i) *N. tabacum* var. *Xanthi* and (ii) *Agrimonia eupatoria*. In the first experimental variation, the *N. tabacum* var. *Xanthi* plants (planted in 0.4 L pots) were watered with 120 mL water solution containing acids (**1–3**) or esters (**1a–1j**, **2a–2j**, **3a–3f**, **3i**, **3j**) at a concentration of 500 mg L^{−1}. Seven days after treatment, visual phytotoxic effects were observed on the plants (e.g., yellowing leaves). In the second experimental variation of phytotoxicity experiments, the *Agrimonia eupatoria* seeds were grown on pans in a water solution with acids (**1–3**) or esters (**1–3**, **1a–1j**, **2a–2j**, **3a–3f**, **3i**, **3j**) at two concentrations: (i) 250 mg L^{−1} and (ii) 500 mg L^{−1}. After 5 days, the sprouts were weighed. Reference samples were grown on pans in water.

2.2.2 SAR induction activity

Plants of *N. tabacum* var. *Xanthi*, at the stage of three-developed leaves, were sprayed with 20 mL solutions of analyzed compounds in water at a concentration of 500 mg L^{−1} and the control sprayed with distilled water that was used for the preparation of water solutions of acids (**1–3**) or esters (**1a–1j**, **2a–2j**, **3a–3f**, **3i**, **3j**). After 7 days, the treated plants were infected mechanically with TMV. The tested plants were evenly dusted with carborandum and gently rubbed with fingers dipped in TMV inoculum. After inoculation, plants were rinsed gently with a stream of water to remove extra inoculum and carborandum. Inoculated plants were kept at 25°C in the light-controlled and insect-free greenhouse. After 4–5 days, the local necrotic spots, as a result of viral infection, were counted and compared between the number of spots on the leaves treated with water solution of the tested substance and distilled water (control). The reduction in the number of necrotic spots on the leaves treated with tested compounds, in comparison with the control, shows inhibition of viral infection by induction of plant resistance through the usage of new compounds. Moreover, aside from the reduction in the number of local necrotic spots, in tobacco plants treated with tested compounds, their size reduction was also observed. The size

of the necrotic area on leaves was determined by using ImageJ software [30]. The surface of the leaf or area with necrotic spots was represented by a set of pixels on the picture, which were counted by the program.

3 Results

We have successfully investigated the biological efficacy of 28 ester derivatives of nicotinic (**1a–1j**), isonicotinic (**2a–2j**), and 2,6-dichloroisonicotinic acid (**3a–3f**, **3i**, **3j**) where three derivatives (**3f**, **3i**, **3j**) were newly synthesized and characterized for the purpose of this article (Figure 1). Compound **3g** was not obtained due to steric hindrance caused by the presence of halogens attached directly to the heterocycle, and **3h** was obtained with monoester derivative as an inseparable mixture. The obtained chemical compounds were characterized using ^1H , ^{13}C NMR, MS, and IR methods. Also, the melting points of solid products were determined. Details of procedures of synthesis and spectra data are reported in the SI.

Most of the ester derivatives presented in this article were known and synthesized before by other groups (**1a–1j**, **2a–2j** and **3a–3e**) [19,31–43]; however, most of them were never tested as plant resistance inducers (except **3a–3c** that were patented as SAR inducers) [19]. Besides the substances mentioned above, we have prepared one new ester (**3f**) and two new diester derivatives (**3i**, **3j**) of 2,6-dichloroisonicotinic acid (Figure 1) and

reported the synthesis, characterization, and biological properties. Furthermore, substances (**1a–1j**, **2a–2j**, and **3a–3e**) were also investigated in our work to illustrate the properties of a whole series of similar derivatives. A family of mono- and diesters of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acids was prepared according to the method described in the literature [44].

Phytotoxicity in plants can occur when certain substances, at a particular concentration, supplied to a plant cause inhibition of plant growth, yellowing of leaves, or even death of the plant. In brief, this parameter could be defined as the degree of toxicity of a chemical compound to plants. In this article, phytotoxicity tests were performed in two variants of the plant model: (1) *N. tabacum* var. *Xanthi* and (2) *Agrimonia eupatoria*.

Although test conditions for seeds and sprouts differ from those for plant tests, they are a relatively quick method to determine whether a tested substance (or environmental conditions) significantly influences plant germination processes. Thus, it is a possible way to determine whether a substance has a phytotoxic effect on the plant. If the substance significantly inhibits seed germination (more than 50% in relation to the control), it could be expected that it will also inhibit the development of plants in the later growth stages.

The first variant of the phytotoxicity test was performed to observe any phytotoxic symptoms caused by the tested substances at concentrations that were later used to evaluate the SAR-inducing properties in plants. In the following experiment, *N. tabacum* var. *Xanthi* plants were watered with 120 mL of a water solution containing ester derivatives

Figure 1: Structures of the investigated compounds: nicotinic acid (**1**), isonicotinic acid (**2**), 2,6-dichloroisonicotinic acid (**3**); monoesters derivatives of nicotinic acid (**1a–1f**), isonicotinic acid (**2a–2f**), and 2,6-dichloroisonicotinic acid (**3a–3f**); diesters derivatives of nicotinic acid (**1g–1j**), isonicotinic acid (**2g–2j**), and 2,6-dichloroisonicotinic acid (**3g–3j**). * Compound **3g** was not obtained due to steric hindrance caused by the presence of halogens attached directly to the heterocycle, and **3h** was obtained with a monoester derivative as an inseparable mixture.

Table 1: Phytotoxic effect and reduction of necrotic areas caused by a viral infection on plants as a result of the application of tested ester derivatives of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acid (500 mg L^{-1} in water, approx. 15 mL per plant via spraying application and approx. 120 mL per plant via watering in phytotoxicity tests on tobacco)

Substance	Phytotoxicity to tobacco	Percent of sprouts mass reduction of <i>Agrimonia eupatoria</i> (%)		Reduction of the necrotic area relative to control plants (%)
		250 mg L^{-1}	500 mg L^{-1}	
1	—	31	36	50 ^e
1a	—	39	55	0 ^h
1b	—	26	46	0 ^h
1c	—	14	33	0 ^h
1d	—	27	47	0 ^h
1e	—	7	30	0 ^h
1f	—	13	35	0 ^h
1g	—	15	38	0 ^h
1h	—	18	52	68 ^d
1i	—	20	33	0 ^h
1j	—	40	42	0 ^h
2	—	54	66	0 ^h
2a	—	57	60	0 ^h
2b	—	53	67	0 ^h
2c	—	63	74	0 ^h
2d	—	3	32	0 ^h
2e	—	40	*	18 ^g
2f	—	5	20	0 ^h
2g	—	50	67	76 ^c
2h	—	40	52	63 ^d
2i	—	41	48	33 ^f
2j	—	22	51	75 ^c
3	—	76	*	82 ^b
3a	—	32	45	0 ^h
3b	—	37	*	60 ^d
3c	—	20	45	21 ^g
3d	—	16	29	82 ^b
3e	—	6	45	93 ^a
3f	—	24	29	0 ^h
3i	—	10	30	97 ^a
3j	—	53	56	93 ^a
Control (H ₂ O)	—	0	0	0 ^h

The presented results are the average of 3 replicates. Control samples were treated only with water. The necrotic area on leaves was determined using ImageJ software. The data were subjected to a one-way analysis of variance (ANOVA). Means with the same letter in superscript (a, b, c, etc.) are not significantly different at $\alpha = 0.05$.

*: no seeds growth; —: does not cause phytotoxic effects on plants.

at a concentration of 500 mg L^{-1} . A control sample was treated only with water. Seven days after the treatment, visual effects caused by the application of tested substances on the plants were analyzed. The test results are shown in Table 1. All the plants were healthy, with no visible yellowing or leaf spots. None of the tested compounds caused phytotoxic effects in tobacco plants, and it can be summarized that the introduction of different ester groups to nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acids does not induce phytotoxic effects.

In the second experimental variation of phytotoxicity experiments, *Agrimonia eupatoria* seeds were grown on Petri dishes and watered with water solution containing acids (1–3) or ester derivatives (1a–1j, 2a–2j and 3a–3j) at two concentrations: (i) 250 mg L^{-1} and (ii) 500 mg L^{-1} . Control samples were grown in water. After 5 days, sprouts were weighed and the obtained results were compared to control samples. The calculated percentage reduction in the weight of sprouts is shown in Table 1. The phytotoxicity of tested acids shows an ascending

order: nicotinic acid (**1**) < isonicotinic acid (**2**) < 2,6-dichloroisonicotinic acid (**3**). The most phytotoxic obtained ester derivatives for *Agrimonia eupatoria* seeds were **1a**, **2a–2c**, **2e**, **2g–2i**, **3a**, **3b**, and **3j**, which showed a weight reduction between 37 and 63% (concentration of the active substance: 250 mg L⁻¹), in comparison to control the sprout mass. The same substances used at a concentration of 500 mg L⁻¹ were more phytotoxic. Furthermore, for **2e** and **3b**, no seed growth was observed. A less phytotoxic effect toward *Agrimonia eupatoria* sprouts was observed with compounds **1c**, **1e**, **1f**, **1i**, **2d**, **2f**, **3d**, **3f**, and **3i**, which caused a decrease in the sprout mass between 20 and 35% at 500 mg L⁻¹ and 3–20% at 250 mg L⁻¹, respectively.

In brief, the obtained results show that ester derivatives of nicotinic acid were more phytotoxic than derivatives of isonicotinic acid. Moreover, modification of 2,6-dichloroisonicotinic acid (**3**) to ester derivatives decreased the phytotoxic effect on *Agrimonia eupatoria* seeds in comparison to the acid.

Plant resistance induction properties of the 28 ester derivatives of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acid were studied based on the inhibition of viral infection. Tobacco plants (*Nicotiana tabacum* var. *Xanthi*) were applied with a water solution of the examined substances (concentration: 500 mg L⁻¹), and, after 7 days, the treated plants were inoculated with the TMV. The control sample was watered only with water and was also inoculated with TMV. Next, the percentage of the total area of necrotic spots caused by virus infection on the leaves was estimated 7 days after infection and compared to control plants (untreated).

The reduction of the necrotic area on leaves was determined by using ImageJ software [30]. The results are shown in Table 1, Figure 2, Table S1, and Figures S85–S88 (Supplementary information).

First, nicotinic (**1**), isonicotinic (**2**), and 2,6-dichloroisonicotinic (**3**) acids were examined toward their biological efficacy. The results showed that the compounds were effective in the following order: 2,6-dichloroisonicotinic acid (**3**) > nicotinic acid (**1**) > isonicotinic acid (**2**), with the plant resistance induction levels being 82, 50, and <10%, respectively. Nevertheless, for further studies, all acids mentioned above were derivatized into the esters and tested using the same conditions. Any reduction of necrotic spots is a criterion for qualifying a compound as active, but only those compounds that show significant biological activity are important from the point of view of research. Only 11 out of the 28 analyzed substances exhibited the plant's resistance induction properties; furthermore, nine (**1h**, **2g**, **2h**, **2j**, **3b**, **3d**, **3e**, **3i**, **3j**) out

Figure 2: Reduction of necrotic areas caused by a viral infection on plants treated with solutions of the best-tested compounds **3e**, **3i**, and **3j** in comparison to control plants (**C**) treated only with water.

of these 11 reduce necrotic areas on leaves caused by viral infection by 50%. The most effective compounds were derivatives of 2,6-dichloroisonicotinic acid; in particular, diesters **3i** and **3j**. After the application of these compounds to plants, the symptoms of viral infection were reduced by up to 97%. In the case of diesters **3i** and **3j**, the plant's natural resistance induction properties were enhanced by 13–18% in comparison to 2,6-dichloroisonicotinic acid. Less effective were monoesters **3d** and **3e**, which use lead to reduce the necrotic area caused by viral infection by 82 and 93%, respectively. Moreover, it was observed that modification of 2,6-dichloroisonicotinic acid to aliphatic monoesters with short chains **3a**, **3b**, and **3c** and a monoester with benzyl ether group **3f** significantly diminish the ability of the elicitor to induce natural plant resistance. Similar relationships were also noticed for nicotinic and isonicotinic acids. None of the monoesters of these acids showed resistance induction properties. In the case of application of isonicotinic diesters **2g**, **2h**, and **2i**, the necrotic area caused by a viral infection on tobacco leaves was reduced by even 75%. As a result of the derivatization of isonicotinic acid into the diester form, it became biologically active (from no observable induction properties of isonicotinic

acid to a level of 75%), whereas, among nicotinic acid derivatives, only diester **1h** showed biological activity of 68%.

In our latest work, we reported that isonicotinic and 2,6-dichloroisonicotinic amide derivatives showed higher SAR-inducing biological activities than acids [29]. In the case of derivatization to monoester derivatives of isonicotinic, nicotinic, and 2,6-dichloroisonicotinic acid, we have observed a reduction of SAR-inducing properties in comparison to acids. In general, diesters of isonicotinic acid and mono- and diesters of INA have significant biological activities, mostly higher than acids.

4 Conclusion

In this study, we have characterized 28 derivatives of nicotinic, isonicotinic, and 2,6-dichloroisonicotinic acids, from which one monoester **3f** and two diesters **3i** and **3j** were newly obtained. None of the obtained substances was phytotoxic to tobacco plants. However, the obtained results show that ester derivatives of nicotinic acid were more phytotoxic to *Agrimonia eupatoria* seeds than derivatives of isonicotinic acid, but a modification of 2,6-dichloroisonicotinic acid to ester derivatives decreased phytotoxicity toward *Agrimonia eupatoria* seeds in comparison to acids. Moreover, the derivatization of 2,6-dichloroisonicotinic acid to ester derivatives decreased phytotoxic effects on *Agrimonia eupatoria* seeds in comparison to acids. Only 11 of the 28 presented substances exhibited resistance induction properties (for compounds **1h**, **2g**, **2h**, **2j**, **3b**, **3d**, **3e**, **3i**, and **3j** reduction of necrotic spots was higher than 50%). Application of these compounds to plant reduced the effects of viral infections even up to 97% (diester derivatives of 2,6-dichloroisonicotinic acid **3i**). In general, modification of nicotinic and 2,6-dichloroisonicotinic acid to monoesters significantly diminished the plants' natural resistance induction properties. Moreover, monoesters of isonicotinic acid did not show resistance induction properties, which indicates that the derivatization of isonicotinic acid to monoesters does not change its biological properties. Whereas, in the case of diesters of isonicotinic acid (**2g**, **2h**, **2i**), the application of these compounds to tobacco plants reduced the number of necrotic spots by 33%. Only one diester of nicotinic acid **1h** had the same biological activity as nicotinic acid (**1**). Other ester derivatives of nicotinic acid (**1a–1g**, **1i**, **1j**) did not have resistance induction properties. It is worth noting, to the best of our knowledge, we have demonstrated for the first time the activity of diesters in inducing

plant resistance. Moreover, we have shown that simple modification of the starting compound can increase the efficiency of biological activity. The obtained results show that tested 2,6-dichloroisonicotinic acid (INA, **3**) has biological activity as SAR inducer at 82%, and its derivatives – monoester **3e**, and diesters **3i** and **3j** investigate higher biological activity (93–97% of plants natural resistance induction) at the tested dose (500 mg L⁻¹) and in the future could be successfully used as new plant resistance inducers.

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