

Coronalon: a powerful tool in plant stress physiology

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Abstract Coralon, a synthetic 6-ethyl indanoyl isoleucine conjugate, has been designed as a highly active mimic of octadecanoid phytohormones that are involved in insect and disease resistance. The spectrum of biological activities that is affected by coronalon was investigated in nine different plant systems specifically responding to jasmonates and/or 12-oxo-phytodienoic acid. In all bioassays analyzed, coronalon demonstrated a general strong activity at low micromolar concentrations. The results obtained showed the induction of (i) defense-related secondary metabolite accumulation in both cell cultures and plant tissues, (ii) specific abiotic and biotic stress-related gene expression, and (iii) root growth retardation. The general activity of coronalon in the induction of plant stress responses together with its simple and efficient synthesis suggests that this compound might serve as a valuable tool in the examination of various aspects in plant stress physiology. Moreover, coronalon might become employed in agriculture to elicit plant resistance against various aggressors.

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Key words: Coralon; Indanoyl isoleucine conjugate; Octadecanoid; Plant stress response

1. Introduction

Lipid-derived signal molecules, such as 12-oxo-phytodienoic acid (OPDA) (Fig. 1, 1) and jasmonic acid (JA) (Fig. 1, 2), are ubiquitously distributed in higher plants. These phytohormones are mainly involved in plant development as well as stress physiology including defense reactions which provide resistance against herbivore or pathogen attack [1,2]. The biosynthesis of these octadecanoids, starting from the fatty acid α -linolenic acid, was elucidated by Vick and Zimmermann [3]. Meanwhile, nearly all enzymes involved in this pathway have been cloned [4]. The phytotoxin coronatine (Fig. 1, 3), an amide of the polyketide coronafacic acid with the rare cyclopropyl amino acid coronamic acid [5], to a certain extent mimics the biological activities of OPDA and/or JA. The responses elicited by these compounds include: tendrils coiling, biosynthesis of terpenoids and other volatiles, expression of

genes in tomato or barley (e.g. jasmonate-induced proteins, JIPs), fruit drop, and accumulation of phytoalexins [6–10]. However, some differences between the effects of coronatine, OPDA, and JA have been well documented in these studies. Coronatine was isolated for the first time from a fermentation broth of the phytopathogenic bacterium *Pseudomonas syringae* var. *atropurpurea* [5,11] but is also produced by several other pathovars of *P. syringae*, e.g. *tomato* and *glycinea*. In addition to the conjugation with coronamic acid, other amino acids have also been found to generate bioactive amides with coronafacic acid, for example norcoronamic acid, L-isoleucine, and L-valine [12].

In comparison to jasmonates, which in some bioassays must be applied in comparatively – for phytohormones – high concentrations (> 200 μ M), coronatine proved to be much more active [7,10,13]. Due to the scientific and economic interest in coronatine's ability to modulate secondary metabolism and elicit plant defense, many groups have attempted to discover an efficient and high-yielding synthetic route to coronatine or structurally related, highly effective compounds. However, the complex stereochemistry of coronatine required labor-intensive and lengthy syntheses. Among the more than 15 synthetic routes that have been published to date, none is short and high-yielding [11].

In previous work, the synthesis of a 6-ethyl indanoyl isoleucine conjugate (2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester) (coralon: Fig. 1, 4), which was designed as a structural mimic of coronatine, has been established [14]. This new coronatine mimic is more effective in various biological assays compared to its simpler indanoyl isoleucine conjugate precursor described earlier [13]. While that precursor also stimulates plant secondary metabolism, its spectrum of biological effects resembles more those responses elicited by JA rather than those elicited by other octadecanoids, resulting in a less broad range of biological responses for the indanoyl isoleucine conjugate than observed for the 6-ethyl-derivative coronalon [8]. As a consequence of this observation, other 6-alkyl indanoyl isoleucine conjugates have been synthesized in addition to coronalon as potential inducers of plant secondary metabolism [8,14,15]. However, coronalon appeared to be the most effective elicitor of certain plant responses, such as volatile biosynthesis in Lima bean, the promotion of fruit and leaf drop in citrus trees, and phytoalexin synthesis and accumulation in soybean [7,8,14].

In the present work, we analyzed the impact of coronalon

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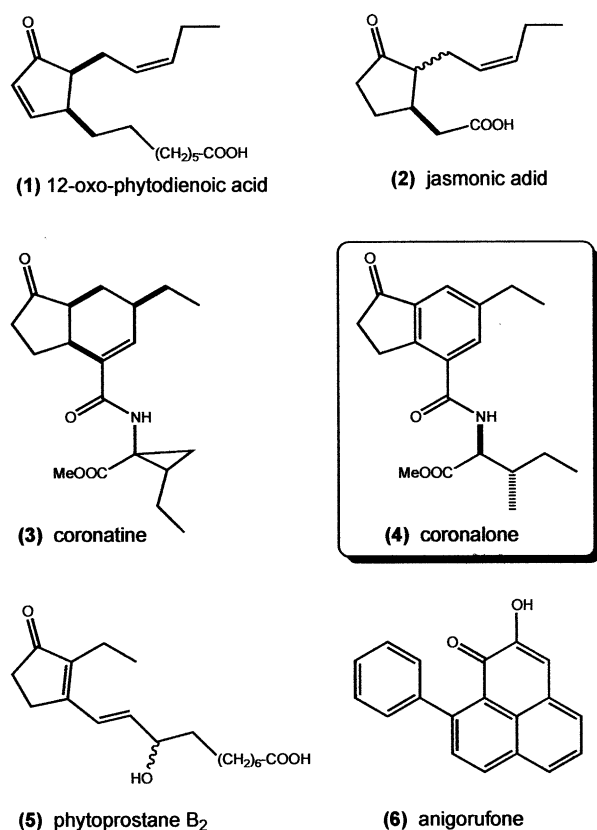


Fig. 1. Structures of octadecanoid-related signaling compounds. (1) OPDA; (2) JA; (3) coronatine; (4) coronalene; (5) phytoprostane B₂; (6) anigorufone.

on seven additional species, including agronomically important plants such as tobacco, tomato, barley, and soybean. We examined a suite of inducible plant responses, which are well known to be important in the stress physiology of higher plants. The high biological activity in the low micromolar range and the general usefulness reveal coronalene to be a valuable and versatile elicitor of secondary metabolism of plants and other responses.

2. Materials and methods

2.1. Salicylate (SA) analysis in *Phaseolus lunatus*

Experiments were carried out using Lima bean, *P. lunatus* ('Ferry Morse' var. *Jackson Wonder Bush*). Individual plants were grown from seeds in a plastic pot (diameter 5.5 cm) with sterilized potting soil at 23°C and 60% humidity using daylight fluorescent tubes at ca. 270 $\mu\text{E}/\text{m}^2/\text{s}$ with a photo phase of 16 h. Experiments were conducted with 12–16-day-old seedlings showing two fully developed primary leaves. These plantlets were cut and placed into a 100 μM coronalene solution. Samples were taken after 0, 7, and 10 h in triplicate. The quantification of endogenous SA by gas chromatography/mass spectrometry was adapted from the protocol of McCloud and Baldwin [16] originally developed for determination of endogenous JA, and performed as described recently [17] using 1 g of treated leaves. The amounts of endogenous SA were calculated from the peak areas of SA methyl ester in comparison to the standard using calibration curves determined independently.

2.2. Benzo[c]phenanthridine alkaloid analysis in *Eschscholzia californica*

Plant cell suspension cultures of *E. californica* were routinely grown in Linsmaier–Skoog medium over 7 days at 23°C on a gyratory shaker (100 rpm) in diffuse light (750 lux) [18]. For induction experiments,

each well of a 24-well plate was inoculated under sterile conditions with 1 ml of a 3–4-day-old *E. californica* cell suspension culture grown under the conditions described above. Each well was treated with 1 μl of coronalene solutions achieving end concentrations ranging from 0.1 to 100 μM . Each dose dependence experiment contained negative controls that comprised cells treated only with the appropriate amount of solvent (EtOH) as used to dissolve the test substance. The multiwell plates were then sealed with parafilm and incubated for 4 days under growth conditions. For the extraction of alkaloids from *E. californica*, the cell suspension culture contained in each well was transferred to a 1.5-ml conical plastic tube. The cells were collected by centrifugation at $14\,000\times g$ for 10 min at room temperature. After removing the aqueous supernatant, 1 ml of an 80% ethanol solution containing 0.1% HCl was added to the pellet. This suspension was incubated for 2 h at 60°C. Insoluble material was removed by centrifugation for 10 min at room temperature and $14\,000\times g$. The clear supernatant was used for spectrophotometric quantitation of benzo[c]phenanthridine alkaloids at 490 nm on a Microplate Reader (MR600, Dynatech).

2.3. Flavonoid analysis in soybean cell cultures

Cell suspension cultures of soybean (*Glycine max* L. cv. Harosoy 63) were grown at 26°C in the dark on a rotary shaker at 110 rpm and were sub-cultured in fresh medium every 7 days [7]. Induction experiments, harvesting, flavonoid extraction, and sample preparation was performed as described [7]. The sample was finally dissolved in 200 μl EtOH and analyzed by high performance liquid chromatography (HPLC) (LiChrosorb RP-18, 4×250 mm; flow rate 1 ml/min; two-step gradient, from 40% to 75% MeOH in 3 min and from 75% to 100% MeOH in 14 min). Compounds were identified and quantified by using reference substances.

2.4. Nicotine analysis in tobacco

Nicotiana attenuata seeds were germinated in soil and grown individually in hydroponic chambers [19]. Plants were treated with 0.6 μM coronalene for 125 h. For nicotine analyses, 30 mg of the ground dry material was extracted in 1.5 ml 40% MeOH, 0.5% acetic acid with shaking (Vortex) for 2 h and subsequently centrifuged for 12 min. Samples were analyzed by HPLC (Inertsil 3 μm , 4.6×150 mm; flow rate 1 ml/min; three-step gradient from 0.25% H_3PO_4 to 12% MeCN in 6 min, then to 18% MeCN in 4 min, and finally to 58% MeCN in 20 min).

2.5. Phenylphenalenone analysis in *Wachendorfia thyrsiflora*

Sterile root cultures of *W. thyrsiflora* were grown as described [20] and treated with 0.8 mM coronalene. After 4 days, roots were harvested, ground in liquid nitrogen and extracted twice with acetone at room temperature for 1 h each. After removing the solvent the samples were dissolved in acetone and analyzed by HPLC (LiChrospher RP-18, 4×250 mm; flow rate 0.5 ml/min; gradient from 30% MeCN to 65% MeCN in 30 min and from 65% to 90% MeCN in 5 min). Compounds were identified and quantified by using authentic reference substances.

2.6. Northern blot analyses in barley and tomato

Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were grown for 8 weeks, mid-sized leaves harvested and treated at 22°C in coronalene solutions according to [9]. After incubation leaves were washed twice with distilled water and frozen in liquid nitrogen until use. RNA extraction (0.5 g fresh weight), electrophoresis (10 μg total RNA per lane), blotting, and hybridization with a ^{32}P -labelled 800-bp fragment of a cDNA encoding proteinase inhibitor 2 of tomato (pin2) were conducted as described [21].

Primary leaves of 6-day-old seedlings of barley (*Hordeum vulgare* cv. Salome) were used to detect the transcripts of the jasmonate-inducible protein of 23 kDa (JIP23), in response to coronalene (0, 5, 10, 21, 50 μM). Incubation was carried out for 24 h in the dark. Growth of seedlings, RNA extraction and Northern blot analysis were performed as described [22]. 10 μg total RNA of leaf segments was loaded per lane.

2.7. Root length assay in *Arabidopsis thaliana*

Sterilized *A. thaliana* seeds (wild-type Columbia and *jin1* mutants [23]) were sown on square agar plates of MS medium supplemented with 1% sucrose, 0.5 g/l MES, and 0.8% Phytigel containing 1 or 10 μM coronalene or 10 μM methyl jasmonate (MeJA). They were

incubated at 4°C for 2 days and placed vertically in a growth chamber at 21°C in continuous light for 10 days.

3. Results

3.1. Coronalon effects on secondary metabolites

3.1.1. Methyl salicylate induction in Lima bean. The treatment of *P. lunatus* with coronalon results in a strong emission of a volatile blend containing methyl salicylate (MeSA) as a very prominent compound [14]. MeSA and its precursor, SA, represent important signal molecules in plant defense reactions, in particular in mediating systemic acquired resistance that is turned on by pathogen attack [24]. Here, the endogenous level of MeSA was determined after 7 h and 10 h upon treatment with a 100 μ M solution of coronalon in Lima bean (Fig. 2A). A nine-fold increase of MeSA within 10 h was observed.

3.1.2. Benzo[c]phenanthridine induction in *E. californica*. Cell suspension cultures of *E. californica* are known to produce brightly red-colored benzo[c]phenanthridine alkaloids when challenged with coronatine or MeJA [10]. A 4-day treatment with various concentrations of coronalon (0.1–100 μ M) showed the strong induction of these alkaloids in a concentration-dependent manner in *E. californica* (Fig. 2B).

3.1.3. 7,4'-Dihydroxyflavone (DHF) induction in soybean. JA, OPDA, and coronatine have been described to cause the synthesis of various flavonoids in soybean cell suspension cultures although the blend of flavonoids is not identical [7,18]. Moreover, the addition of both OPDA and coronalon led to the accumulation of the soybean phytoalexins (glyceollins) which are also elicited by pathogen attack or elicitor treatment [7,25]. Besides glyceollins, a novel flavonoid, DHF, was observed to be induced in a concentration-dependent manner during coronalon treatment for 48 h (Fig. 2C).

3.1.4. Nicotine induction in tobacco. In *N. attenuata* leaves, the level of the toxic alkaloid nicotine increases upon wounding or treatment with jasmonates [26]. Coronalon (100 μ M) was used to analyze its nicotine-inducing activity compared with a water control. The nicotine concentrations in the whole plants were determined to be 1.7 ± 0.7 μ g/mg dry mass in the control and 11.3 ± 1.5 μ g/mg dry mass in the coronalon-treated plants.

3.1.5. Phenylphenalenone accumulation in *W. thyrsoflora*. Phenylphenalenones are secondary metabolites often used for plant taxonomy. Recently, in banana species these compounds have been observed as phytoalexins accumulating upon nematode attack [27]. In this study, coronalon (0.8 mM) treatment elicited the accumulation of the phenylphenalenone, methoxy-anigorufone, in *W. thyrsoflora* cultured roots from 1.9 μ g/g fresh weight in the untreated control up to 16.3 μ g/g fresh weight.

3.2. Induction of gene expression in barley and tomato

In order to assess the impact of coronalon on gene expression, two different stress-related genes were analyzed by Northern blot analysis in this study. Upon hyperosmotic stress, wounding or challenge with JA, barley synthesizes certain proteins referred to as JIPs [28]. JIP23 is a typical, strongly JA-induced protein of still unknown function. To investigate the coronalon activity on inducing JIP23 gene expression in barley seedlings, segments of primary leaves of 6-day-old seedlings of barley were floated on aque-

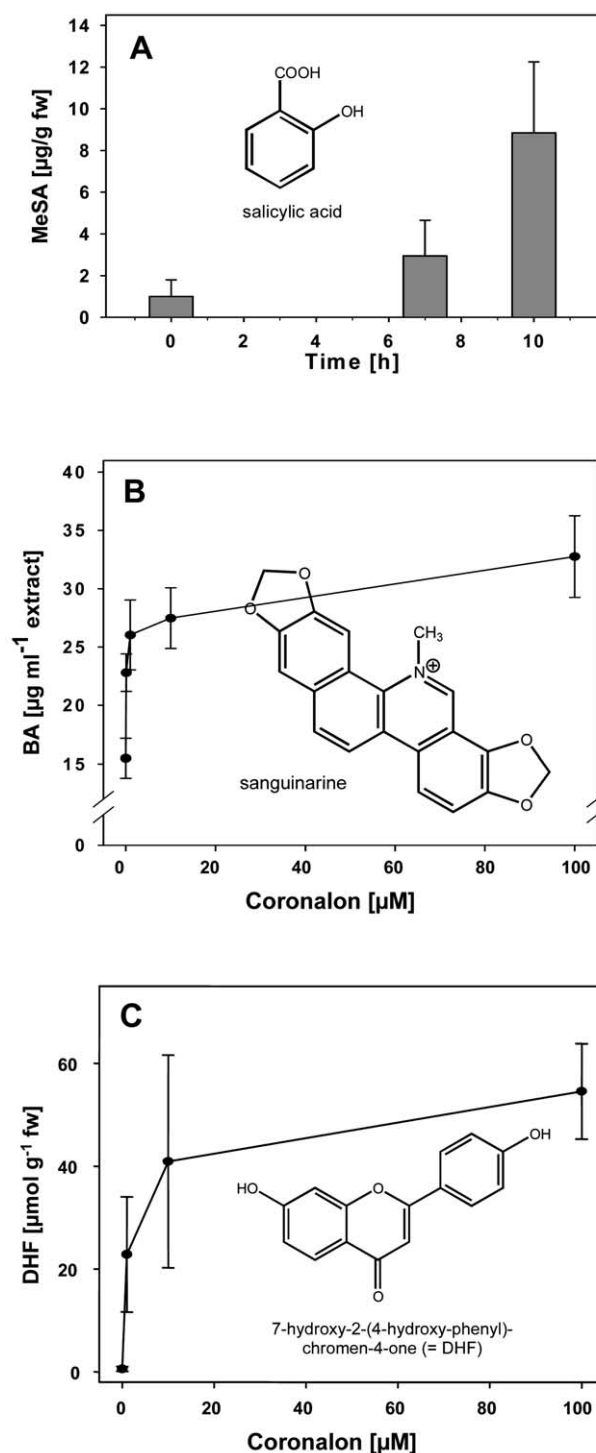


Fig. 2. Induction of secondary metabolite accumulation in various plants upon treatment with coronalon. A: Time-dependent induction of salicylic acid in Lima bean leaves. Samples were taken at the beginning (0 h), 7 h and 10 h after elicitation with coronalon (100 μ M). The salicylate level at $t=0$ represents the resting level. B: Coronalon concentration-dependent induction of benzo[c]phenanthridine alkaloids in *E. californica* cell suspension cultures. Sanguinarin is shown as a representative of the family of benzo[c]phenanthridine alkaloids. C: Coronalon concentration-dependent induction of 7,4'-dihydroxyflavone in soybean cell suspension cultures. The results are the mean \pm S.D. ($n \geq 3$).

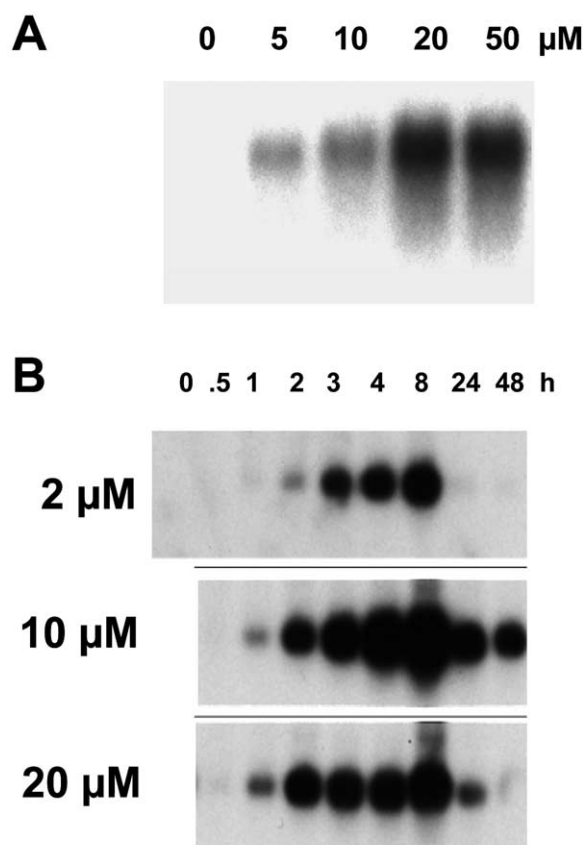


Fig. 3. Northern analyses of two jasmonate-inducible genes. A: Concentration-dependent effect of coronalon on the transcript level of *JIP23* in barley leaves. B: Concentration- and time-dependent effects of coronalon on the mRNA expression level of *Pin2* in tomato leaves. 10 µg total RNA was added per lane.

ous solutions containing 5–50 µM coronalon and analyzed after 24 h of incubation. As demonstrated in Fig. 3A, *JIP23* gene expression was clearly up-regulated and the corresponding mRNA accumulated even at the lowest coronalon concentration applied.

In tomato plants, *pin2* mRNA accumulation was identified as a well-known wound response marker [29]. Leaves were cut at the petioles and floated on aqueous solutions containing 2, 10, or 20 µM coronalon. Samples were taken after several time intervals ranging from 0 to 48 h for Northern blot analysis. A strong, fast, and transient induction of *pin2* transcripts

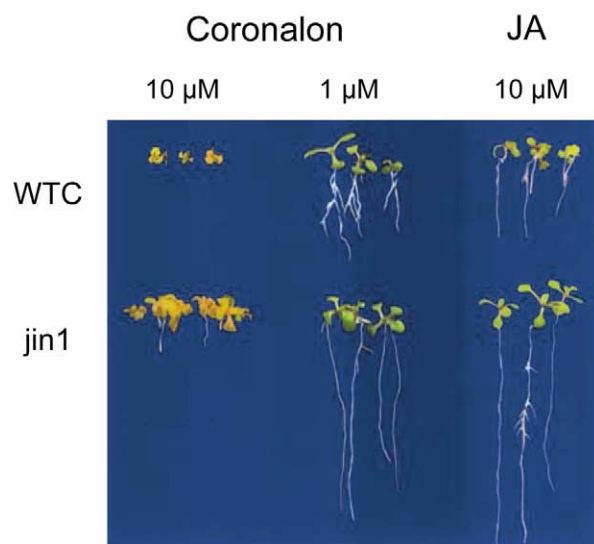


Fig. 4. Effects of coronalon and MeJA on the root growth of *A. thaliana* seedlings. Wild-type seeds (WTC) and *jin1* mutant seeds which were less sensitive to JA were treated with coronalon or MeJA for 2 days. After 10 additional days of incubation the root lengths of the particular seedlings were determined.

was observed at all concentrations tested (Fig. 3B). Interestingly, the induction pattern obtained with 10 µM coronalon was both longer-lasting and stronger compared with the induction obtained with 20 µM or 2 µM inducer. This result suggests an optimum in the dose–response relationship.

3.3. Root growth retardant activity in *A. thaliana*

The presence of JA and MeJA has a strong impact on the root growth in *A. thaliana* seedlings, i.e. JA-treated seedlings have shorter and more branched roots [23]. This effect has been used to isolate mutants with lower sensitivity to MeJA. One of these JA-insensitive mutants, *jin1*, and the corresponding wild-type were used to compare the influence of coronalon with that of MeJA. As expected, a difference in root length was observed in the wild-type versus the *jin1* mutant when treated with 10 µM MeJA (Fig. 4). Treatment with 1 µM coronalon showed an effect comparable with that obtained by MeJA application in both plants. However, using 10 µM coronalon the root growth in wild-type was almost completely inhibited and also the *jin1* mutant showed a strongly reduced root growth (Fig. 4).

Table 1
Survey of coronalon-induced reactions in various plant species

Species	Induction	Physiological relevance	Reference
<i>Phaseolus lunatus</i>	Volatiles	Herbivore attack	[14]
<i>Medicago truncatula</i>	Volatiles	Herbivore attack	Unpublished result
<i>Glycine max</i> ^a	Glyceollins	Pathogen challenge	[7]
	DHF	Flower color	This work
<i>Bryonia dioica</i>	Tendrils coiling	Touching	[8,14]
<i>Citrus sinensis</i>	Fruit, leaf drop	Senescence	[8]
<i>Nicotiana attenuata</i>	Nicotine	Herbivore attack	This work
<i>Eschscholzia californica</i> ^a	Benzo[c]phenanthridines	Elicitation	This work
<i>Wachendorfia thyrsiflora</i> ^b	Phenylphenalenones	Nematode infection	This work
<i>Lycopersicon esculentum</i>	Proteinase inhibitor-2	Herbivore attack	This work
<i>Hordeum vulgare</i>	JIP23	Osmotic stress	This work
<i>Arabidopsis thaliana</i>	Root growth inhibition	Root development	This work

^aCell suspension culture.

^bRoot culture.

4. Discussion

Linolenic acid-derived octadecanoids such as OPDA and JA are widely distributed phytohormones in higher plants with a broad spectrum of biological effects in plant development, insect and disease resistance, and stress physiology [1,2]. Exogenous application of octadecanoids induces specific stress-responsive genes as well as the synthesis and accumulation of various secondary metabolites, including defense-related compounds. Many of these plant responses can also be elicited by the bacterial phytotoxin coronatine [8,10]. Based on the structure of coronatine, 6-substituted indanoyl isoleucine conjugates have been synthesized in order to develop synthetically produced compounds with similar or even identical biological activities compared to coronatine and the octadecanoids [13–15,30]. As outlined recently by Lauchli and Boland [8], one advantage of these compounds over other signaling molecules lies in the chemistry of the indanoyl amino acid conjugates which allow an easy synthesis of related compounds that can be tuned for particular requirements and biological activities. The 6-ethyl indanoyl isoleucine conjugate, coronalon, proved to fulfill the expected standards when applied to induce tendril coiling on *Bryonia dioica* or the induction of secondary metabolite accumulation [7,13–15]. These results encouraged us to further investigate the spectrum of possible physiological activities of coronalon with a focus on typical octadecanoid-inducible responses. In addition to their ability to elicit a broad range of inducible biological responses, jasmonates and OPDA revealed some diversity in their particular activity patterns suggesting that these signaling molecules are involved in different physiological signaling processes [31,32]. Coronalon, however, seems to combine the activity profiles of the two groups of octadecanoids. For example, the synthesis of DHF (Fig. 2C) and glyceollin in soybean is mainly induced by the early octadecanoid OPDA [7,33], while, in contrast, nicotine synthesis and *JIP23* gene expression (Fig. 3A) are typically jasmonate-triggered reactions [26,28].

In the present study, we chose economically important crops such as tobacco, tomato, soybean, barley, and Lima bean to investigate the responsiveness of these plants to coronalon treatment as a prerequisite for any possible applications in field studies or as elicitors of phytoalexin biosynthesis in cell cultures. The activity of coronalon in all these plant species belonging to various plant families as well as in differentiated tissue and cell cultures (soybean, *E. californica*, *W. thyrsoiflora*) indicates an extremely high impact of this compound on higher plants (Table 1) that is at least comparable with the octadecanoid phytohormones but probably more general. As suggested by some preliminary results (data not shown), coronalon even mimics further oxygenated derivatives of polyunsaturated fatty acids present in a complex mixture of signaling compounds referred to as oxylipins [34], including the recently discovered phytosteranes (e.g. Fig. 1, 5) [35]. The latter share with the indanoyl isoleucine conjugates the planar structure of the central ring system. Because oxylipins are believed to be involved in host responses to hostile invaders and herbivores and coronalon indeed efficiently induced accumulation of phytoalexins and defense-related compounds in various plants (Figs. 2 and 3A), it might be employed to prepare and strengthen plants for aggressors.

Although the role of octadecanoids is often regarded as

mainly defense-related, these chemical signals certainly fulfill a broader range of functions in plant stress physiology and development [1,2]. Thus, the effects of coronalon were investigated on the root growth of *A. thaliana* seedlings and osmotic stress response in barley. This latter abiotic stress – as well as jasmonates – induces the expression of a specific gene, *JIP23* [28]. As shown in Fig. 3B, coronalon was efficient in the induction of *JIP23* mRNA accumulation also, substituting the stress factor. Furthermore, on wild-type *A. thaliana* plants, coronalon at a concentration of 1 μ M had the same inhibitory effect on root growth as MeJA (10 μ M) (Fig. 4) confirming its high activity. Moreover, on *A. thaliana* mutants (*jin1*) that are less sensitive to jasmonates [4], coronalon again resembled MeJA activity (Fig. 4) suggesting that both signaling compounds initiate the same signaling pathway.

The extreme potency of coronalon (Table 1) and its derivatives may enhance their value as agrochemicals; they need only to be applied in very small doses to elicit an effect, a trait of both economic and environmental advantage. However, the specificity of these compounds is not yet entirely understood and side effects, both desirable and undesirable, have to be expected. A complete understanding of their physiological effects is of prime importance and might aid in the design of substances with specificity, potency and persistence tailored to particular needs.

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