



# Neomycin: An Effective Inhibitor of Jasmonate-Induced Reactions in Plants

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## Abstract

Jasmonates are important phytohormones involved in both plant developmental processes as well as defense reactions. Many JA-mediated plant defense responses have been studied in model plants using mutants of the jasmonate signaling pathway. However, in plant species where JA-signaling mutants are not accessible, the availability of a tool targeting JA signaling is crucial to investigate jasmonate-dependent processes. Neomycin is a poly-cationic aminoglycoside antibiotic that blocks the release of  $\text{Ca}^{2+}$  from internal stores. We examined the inhibitory activities of neomycin on different jasmonate-inducible responses in eight different plant species: Intracellular calcium measurements in *Nicotiana tabacum* cell culture, Sporamin gene induction in *Ipomoea batatas*, *PDF2.2* gene expression in *Triticum aestivum*, Nepenthesin protease activity measurement in *Nepenthes alata*, extrafloral nectar production in *Phaseolus lunatus*, nectary formation in *Populus trichocarpa*, terpene accumulation in *Picea abies*, and secondary metabolite generation in *Nicotiana attenuata*. We are able to show that neomycin, an easily manageable and commercially available compound, inhibits JA-mediated responses across the plant kingdom.

**Keywords** Jasmonate · Inhibitor · Defense · Dicots · Gymnosperms · Monocots · Neomycin · Signaling

## Introduction

Jasmonate phytohormones play important roles in different plant developmental processes as well as in defense reactions against necrotrophic fungi and insect herbivores throughout the plant kingdom (Wasternack 2007; Wasternack and Hause 2013). Their biosynthesis starts from linolenic acid

and generates the bio-active form of jasmonic acid (JA), (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), in addition to several other jasmonates and metabolites whose activity is less well understood. JA-Ile is generated in the cytosol by the conjugation of JA with isoleucine catalyzed by JAR1 (Staswick and Tiryaki 2004) and interacts with the  $\text{SCF}^{\text{COI1}}$ -JAZ co-receptor complex in the nucleus. Nuclear JAZ proteins, repressors of jasmonate-regulated gene transcription, are subsequently ubiquitinated and targeted for 26S proteasome-mediated degradation, thereby activating jasmonate-responsive genes (Wasternack and Hause 2013; Chini et al. 2007;

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Thines et al. 2007). This interaction is potentiated by inositol pentakisphosphate (IP<sub>5</sub>) (Sheard et al. 2010).

For plants it is well established that upon herbivore attack, changes in cytosolic Ca<sup>2+</sup> concentrations, [Ca<sup>2+</sup>]<sub>cyt</sub>, are prerequisite for the activation of downstream jasmonate signaling (Maffei et al. 2007; Arimura et al. 2008; Bricchi et al. 2010). The increase in [Ca<sup>2+</sup>]<sub>cyt</sub> is due to an influx of Ca<sup>2+</sup> ions from the apoplast, from intracellular compartments, or from both (Lecourieux et al. 2006). Neomycin is a poly-cationic aminoglycoside antibiotic that blocks the release of Ca<sup>2+</sup> from internal stores. Its antibiotic properties against bacteria are due to its ability to interact with prokaryotic 16S rRNA, thereby affecting ribosomal function and protein synthesis (Moazed and Noller 1987; Woodcock et al. 1991). In eukaryotic cells, neomycin is described as a non-specific inhibitor of phospholipase C that finally affects the formation of inositol trisphosphate (IP<sub>3</sub>). IP<sub>3</sub>, in turn, is a well-characterized effector of Ca<sup>2+</sup> release from internal stores in animal cells; in plant cells, both IP<sub>3</sub> and IP<sub>6</sub> are effective (Lemtiri-Chlieh et al. 2003; Peiter 2011). Another role for IPs is emerging in jasmonate perception, since IP<sub>5</sub> binding was shown to be crucial for the COI1-JAZ co-receptor complex to perceive JA-Ile with high sensitivity (Sheard et al. 2010; Mosblech et al. 2011).

Effects of neomycin have been described in different plant species. For example, neomycin inhibited [Ca<sup>2+</sup>]<sub>cyt</sub> elevations that were induced by various elicitors in *Nicotiana tabacum* (cultivated tobacco) and *Glycine max* (soybean) suspension cells (Mithöfer et al. 1999; Cessna et al. 2007; Vatsa et al. 2011); whereas neomycin inhibited IP<sub>3</sub> increases and Ca<sup>2+</sup> transients in *Papaver rhoeas* (common poppy) and *Arabidopsis thaliana* (Arabidopsis) (Franklin-Tong et al. 1996; Tang et al. 2007). Moreover, neomycin has also been shown to regulate a non-selective voltage-dependent cation channel in the vacuolar membrane of plant species, the slow vacuolar (SV) channel (Scholz-Starke et al. 2006), also reported as the TPC1 channel (Peiter et al. 2005). Recently, it has been shown in *A. thaliana* that neomycin affected the accumulation of both [Ca<sup>2+</sup>]<sub>cyt</sub> and JA-Ile when plants were induced with oral secretions from herbivorous lepidopteran *Spodoptera littoralis* larvae. In addition, neomycin treatment reduced the downstream expression of some JA-responsive genes, *VSP2* and *LOX2* (Vadassery et al. 2014), in agreement with the known connection between [Ca<sup>2+</sup>]<sub>cyt</sub> elevation and jasmonate signaling.

Many JA-mediated plant defense responses have been successfully studied in *A. thaliana* and other plants using mutants of the jasmonate signaling pathway. Jasmonate biosynthesis mutants including *jar1* or jasmonate insensitive *coi1* mutants have been extremely helpful to understand JA signaling (Wasternack and Hause 2013). The *jar1* mutant is of particular interest because JAR1 directly generates JA-Ile (Staswick and Tiriyaki 2004). However,

there are still only few plant species where such mutants or transgenic RNA interference (RNAi) plants are available and characterized: the dicotyledonous plants *A. thaliana* (Staswick et al. 1992), *Nicotiana attenuata* (Kang et al. 2006), *Solanum lycopersicum* (tomato) (Suza et al. 2010), and the monocotyledonous *Oryza sativa* (rice) plant (Riemann and Takano 2008). For all plant species where JA-signaling mutants are not accessible or where transgenic approaches are not practical, the availability of a tool affecting directly JA signaling would be an important alternative in order to investigate jasmonate-dependent processes in plants.

Recently, two different inhibitors of jasmonate signaling have been developed. Jarin-1 is a cytosine alkaloid derivative featuring a 3-aminocytosine residue linked to biphenylcarboxylic acid and 3-methoxypropionic acid (Meesters et al. 2014). Jarin-1 was identified from screening a library of natural and semisynthetic compounds. In addition, a derivative of the bacterial JA-Ile mimic coronatine, coronatine-*O*-methyloxime, was de novo designed and synthesized (Monte et al. 2014). Whereas coronatine-*O*-methyloxime prevents the COI1-JAZ interaction, jarin-1 affects jasmonate responses by inhibiting the JA-Ile-generating enzymatic activity of JAR1 (Monte et al. 2014; Meesters et al. 2014). For both compounds, their inhibitory activities have been shown in detail for *A. thaliana* plants. The drawback of these compounds is their limited availability for a broad plant science community because they are neither commercially available nor easy to synthesize.

Neomycin has the potential to act as an inhibitor of jasmonate-mediated responses, is widely available, and usable by researches that are limited by financial constraints. We examined the inhibitory activities of neomycin on different jasmonate-inducible responses in eight different plant species. This survey covers dicots, monocots, and gymnosperms. The broad suitability in planta reveals that neomycin could become a valuable, easily manageable, and commercially available tool to examine jasmonate-dependent effects in the plant kingdom.

## Materials and Methods

### Chemicals

Neomycin sulfate was purchased either from Enzo Life Science (Lörrach, Germany), Duchefa Biochemie (Haarlem, The Netherlands) or Sigma-Aldrich (Darmstadt, Germany) and dissolved in water. Jasmonic acid methyl ester (MeJA) was from Sigma-Aldrich (Darmstadt); jasmonic acid (JA) was synthesized from MeJA by saponification.

## Intracellular Calcium Measurements in *Nicotiana tabacum* Cell Culture

Measurements of intracellular calcium responses were performed on tobacco BY-2 suspension cells (*N. tabacum* L. cv Bright Yellow BY-2) constitutively expressing aequorin in the cytosol (Pauly et al. 2001).

For cytosolic calcium measurements, BY-2 cells were harvested between 60% and 70% of packed cell volume (PCV) and were washed twice by 4 volumes of a minimal buffer (2 mM Mes-KOH pH 5.8, 175 mM Mannitol, 0.5 mM K<sub>2</sub>SO<sub>4</sub> and 0.5 mM CaCl<sub>2</sub>). Cells were adjusted to 20% of PCV and incubated with 2.5  $\mu$ M native coelenterazine overnight before analyses. Calcium measurements were performed using a Sirius Luminometer (Titertek-Berthold, Pforzheim, Germany) in triplicate on 100  $\mu$ L of cells, and the mean trace of the three replicates is presented with standard error bars except for water treatment. For neomycin inhibition, cells were incubated with 100  $\mu$ M of neomycin 20 min before JA stimulation. Calibration was performed after lysis of cells to discharge the whole stock of functional aequorin as previously described (Knight et al. 1996).

## Analysis of *SPORAMIN* Gene Induction by qPCR in *Ipomoea batatas*

Sweet potato (*I. batatas* cv. Tainong 57) plants were grown as described until at least 5 fully expanded leaves were present (Rajendran et al. 2014). The second youngest fully developed leaf was taken for treatment and analyses. The leaves were cut and immersed in 1 $\times$ MS with or without 100  $\mu$ M neomycin for 12 h prior to spraying 50  $\mu$ M MeJA. After 1 h, leaves were harvested for further analysis. All conditions for RNA extraction, qRT-PCR, and primer sequences for Sporamin and for the reference gene ACTIN were taken from (Rajendran et al. 2014).

## Analysis of *Triticum aestivum* PDF2.2 Gene

Wheat (*Triticum aestivum*) cv Yangmai #6 plants were grown under controlled conditions at a photoperiod of 16-h (25 °C) light and 8-h dark (22 °C) at a relative humidity of 73–75% at IISER-Kolkata. Fertilizer was supplied to the plant as nitrogen, phosphorus, and sulfur (NPK) at a ratio of 120:60:40. At growth stage 59 (GS59; Zadok's growth stage) when the ear had fully emerged and flag leaf had expanded, plants were subjected to a foliar spray of 100  $\mu$ M neomycin followed by JA (10  $\mu$ M) after neomycin run-off; or, as a control, water was applied followed by JA (10  $\mu$ M) foliar spray. Flag leaves were collected at 0, 0.5, 1.0, or 1.5 h

post spray (hps) into liquid nitrogen and kept at –80 °C until further processing.

Total RNA was extracted using Trizol (Thermo Fisher Scientific), and 5  $\mu$ g of total RNA was then reverse-transcribed using the superscript first strand cDNA synthesis kit and oligo-dT primers (Invitrogen) according to the manufacturers' instructions. SYBR green assays were performed using the "Power SYBR green" kit (Life Technologies). Gene-specific primers for *PDF2.2* and *Tubulin* were used for normalization in each sample as previously described (Sahu et al. 2016). cDNA templates corresponding to 50 ng of total RNA before reverse-transcription were used in the quantitative real-time PCR (qPCR) assay. The double-delta CT ( $\Delta\Delta$ CT) method was used for data analysis (Sahu et al. 2016). Transcript levels in plants corresponding to time point 0 (controls) were set to 1 (as reference) for relative transcript abundance of the gene *PDF2.2* at the following time points described. Three to four biological replicates were used.

## Nepenthesin Protease Activity Measurement in *Nepenthes alata*

Two-week-old closed pitchers of *N. alata* were induced by injection with a syringe (Buch et al. 2015). After 1 week of treatment with water (control), JA (200  $\mu$ M final concentration), neomycin (100  $\mu$ M final concentration), and neomycin followed by JA 6 h later, nepenthesin proteolytic activity was measured in the digestive fluid of the pitchers using the specific FRET-based substrate, PFU-093, as previously described (Buch et al. 2015). Briefly, 50  $\mu$ L pitcher fluid, 39  $\mu$ L water, and 1  $\mu$ L PFU-093 substrate (80  $\mu$ M) were mixed in black 96-well microtiter plates (Greiner Bio-one GmbH, Frickenhausen, Germany). After incubation for 5 h at room temperature, 10  $\mu$ L of 100 mM Tris-HCl buffer, pH 8.5, was added to the mixture. The fluorescence of cleaved substrate was measured at 42 °C with a microplate reader (Tecan infinite M200, Männedorf, Switzerland) using the excitation/emission wavelengths of 485 nm/530 nm, respectively.

## Quantification of Extrafloral Nectar in *Phaseolus lunatus*

Lima bean (*P. lunatus*) growth, treatment, and the determination of EFN were performed as described by Radhika et al. (2010). Plants were treated (sprayed) with either water, Tween-20 (0.1%), JA (1 mM), neomycin (500  $\mu$ M), or neomycin (500  $\mu$ M) + JA (1 mM). The relatively high concentrations of JA and neomycin were adapted from Radhika et al. (2010) and Tween-20 was added to increase the permeability of neomycin in lima bean leaves. The EFN was quantified

as the amount of soluble solids per gram dry weight of leaf material secreted in 24 h.

### Analysis of Nectary Formation in *Populus trichocarpa*

Plant material was derived from 4-week-old *Populus trichocarpa* (black cottonwood) trees in Clackamas County, OR, USA. We selected 11, spatially separated plant individuals for production of clones showing conformity in size (2–3 m in height). Stem cuttings (60 cm long, one cutting per plant) were removed from the main shoot 70 cm down the apex, immediately placed in water and transported to the laboratory. For plant cultivation, in the laboratory stem cuttings were divided into four 15-cm-long shoot segments, each placed in a glass flask filled with sterilized tap water to an immersion depth of 6 cm. Flasks were covered with aluminum foil to induce root development in the dark. When cuttings had formed roots of about 1 cm in length, they were transferred into 15 cm round plastic pots filled with a 1:1 ratio of standard substrate (Fox Farms, Arcata, CA, USA) and sand (grain size 0.5–2.0 mm). All plants were fertilized with 50 mL of a 0.1% aqueous solution of Shultz All Purpose Plant Food® [NPK(%); 15, 10, 15-Fertilizer] once a week and watered daily. Plants were cultivated in a greenhouse with a light regime of 13-h light (23 °C) and 11-h dark (18 °C) and relative air humidity adjusted to 50–60%. Position of plants in the greenhouse was changed every 3 days to exclude any position effects. Neomycin and JA treatments were applied when plants had developed three new leaves (after about 4 weeks) at which time the percentage of leaves with nectaries per plant was recorded. After 6 weeks, the percentage of newly formed leaves with nectaries per plant was evaluated for all treatments. For treatment, plants were sprayed with 200 µM aqueous JA solution (30 min after the start of the light period at 9:00 a.m.) until the upper and lower sides of all leaves were completely wet (15 mL per plant). Neomycin (100 µM; 15 mL per plant) was sprayed 6 h prior to JA treatment in the dark. Control plants were sprayed with water (control 1) or neomycin alone (control 2).

### Determination of Terpene Accumulation in *Picea abies*

Clones from a single *Picea abies* (Norway spruce) line were treated with MeJA (100 µM), neomycin (100 µM), and MeJA plus neomycin (1 × and 3 × over a period of 2 months). Tween 20 was used in the spray solvents to help the treatments penetrate the trees' waxy surfaces. Samples (bark tissue from 3-year-old trees) were collected into liquid nitrogen and stored at −80 °C. The analysis protocol was adapted from Lewinsohn et al. (1993). Briefly, 100 mg from each sample was ground under liquid nitrogen using a

mortar and pestle and extracted under continuous shaking for ~20 h in 1 mL of TBME containing both 10 µg mL<sup>−1</sup> 1,9-decadiene (Sigma-Aldrich) and 11.5 µg mL<sup>−1</sup> dichlorodehydroabietic acid (CanSyn, Toronto, CAN) as internal standards. The extract was removed, washed with 0.4 mL of 0.1 M (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, pH 8.0, and subsequently dried by running through a Pasteur pipette filled with 0.5 mg Na<sub>2</sub>SO<sub>4</sub>. The Na<sub>2</sub>SO<sub>4</sub>-containing column was further washed with 1 mL of TBME. For methylation of diterpenoid resin acids, 50 µL of TMSH was added to 0.4 mL of extract. Diterpenoids were subsequently analyzed by gas chromatography (GC) coupled to either mass spectrometry (MS) or flame ionization detection (FID); the remainder was used for monoterpene analysis. GC conditions were used as described by Schmidt et al. (2011).

### Analyses of Secondary Metabolites in *Nicotiana attenuata*

Germination and growth of *N. attenuata* (wild tobacco) plants was as previously described (Krügel et al. 2002; Schuman et al. 2014). The youngest fully expanded rosette leaf (position + 1) on intact rosette-stage plants was wounded using a tracing wheel to make 6 rows of puncture wounds (3 on either side of the midvein) to the adaxial surface, and 20 µL of one of the following was immediately added to wounds: water (W + W), neomycin (100 µM) followed 15 min later by water (W + W + N), *Manduca sexta* oral secretions (W + OS), or neomycin followed 15 min later by oral secretions (W + OS + N); control leaves were left untreated (*n* = 6). Leaves were harvested after 3 days to liquid nitrogen. Metabolites were extracted from ground leaf tissue in acidified 40% methanol and profiled by liquid chromatography–time-of-flight mass spectrometry (LC–ToFMS, Bruker) in positive mode as previously described (Kim et al. 2011). As a quality control measure, one of the most concentrated samples was run in dilution series and several compounds were checked for a linear regression with dilution; additionally, external standards containing nicotine and caffeoylputrescine at a representative range of concentrations were analyzed and found to be within a linear range.

## Results

### Effect of Neomycin on Jasmonate-Induced Intracellular Calcium Levels

Specific intracellular changes of Ca<sup>2+</sup> concentrations in the cytosol of plant cells are described upon challenge with pathogen-derived elicitors, herbivory-related stress or direct treatment with jasmonates (Maffei et al. 2007; Walter et al. 2007). In aequorin-expressing tobacco BY2 cell

suspension cultures, the application of JA induced a rapid transient increase of the cytosolic  $\text{Ca}^{2+}$  concentration while in the control cells, no change of cytosolic  $\text{Ca}^{2+}$  was measured (Fig. 1). This JA effect was reduced in the presence of 100  $\mu\text{M}$  neomycin (Fig. 1).

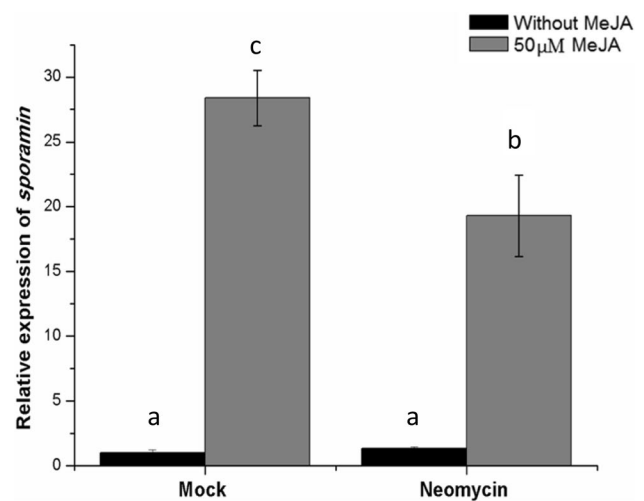
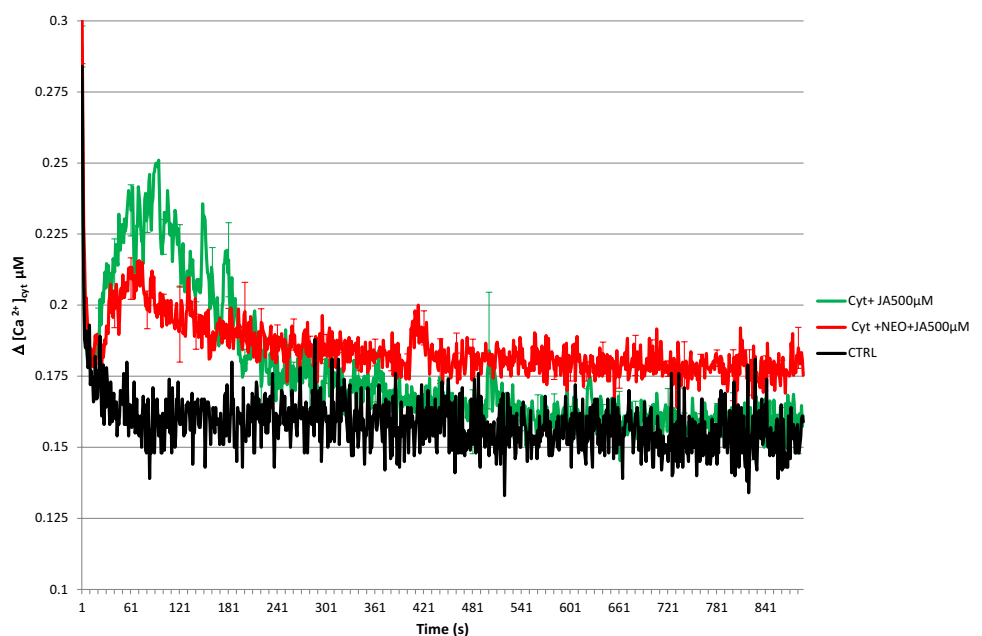
### Effect of Neomycin on Jasmonate-Induced Defense-Gene Expression

The induction of defense-related genes upon JA treatment is well established for numerous plant species (Wasternack 2007; Wasternack and Hause 2013). We analyzed both a dicotyledonous (sweet potato) as well as a monocotyledonous (wheat) species for jasmonate-mediated gene induction. In sweet potato, the JA-induced mRNA level for the defense protein sporamin was not completely, but significantly reduced when plants were also treated with neomycin (Fig. 2). In wheat, the result was the same; compared to the control, the presence of neomycin inhibited the JA-induced mRNA accumulation of the tested gene, *TaPDF2.2* (Fig. 3).

### Effect of Neomycin on a Jasmonate-Induced Protease, Nepenthesin

Nepenthesin is the dominating protease in the digestive fluids of the carnivorous pitcher plants of the genus *Nepenthes*. This protease was shown to be JA-inducible, both on the mRNA and on the enzyme activity level (Buch et al. 2015; Yilamujiang et al. 2016). Here, the JA-induced increased proteolytic activity of nepenthesin was measured in the presence and absence of neomycin in pitcher fluid of *Nepenthes*

**Fig. 1** Neomycin treatment inhibits jasmonate-induced cytosolic calcium transient. Monitoring of cytosolic calcium changes in tobacco (*Nicotiana tabacum*) BY-2 cells; induction was done with 500  $\mu\text{M}$  JA, neomycin was used at 100  $\mu\text{M}$ . Traces correspond to the mean trace of 3 replicates  $\pm$  standard error. Neomycin inhibitory effect is significant at  $P < 0.05$  ( $t$ -test)



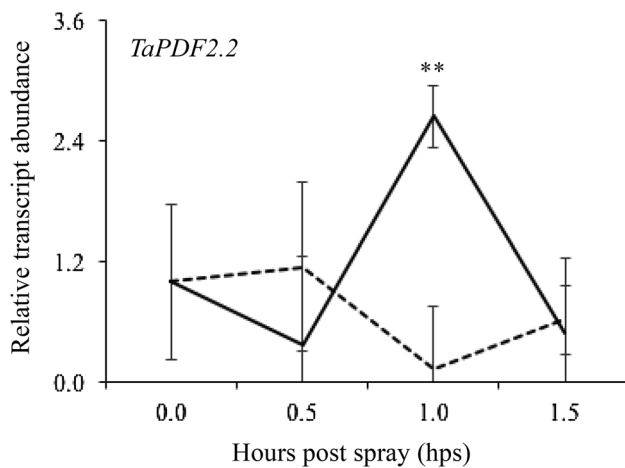
**Fig. 2** Neomycin treatment affects MeJA-induced *SPORAMIN* accumulation in *Ipomoea batatas* (sweet potato). Leaves were cut with the petiole and immersed in  $1\times\text{MS}$  with/without 100  $\mu\text{M}$  neomycin for 12 h prior to application (spraying) of 50  $\mu\text{M}$  MeJA; after 1 h of MeJA treatment, the expression levels of *SPORAMIN* gene transcripts were quantified by qRT-PCR ( $\pm\text{SD}$ ,  $n=5$ ). Different letters indicate significant differences among treatments ( $P < 0.05$ ) in an SNK post hoc test following a 1-way ANOVA

*alata*. As shown in Fig. 4, the presence of 100  $\mu\text{M}$  neomycin reduced the measurable protease activity significantly.

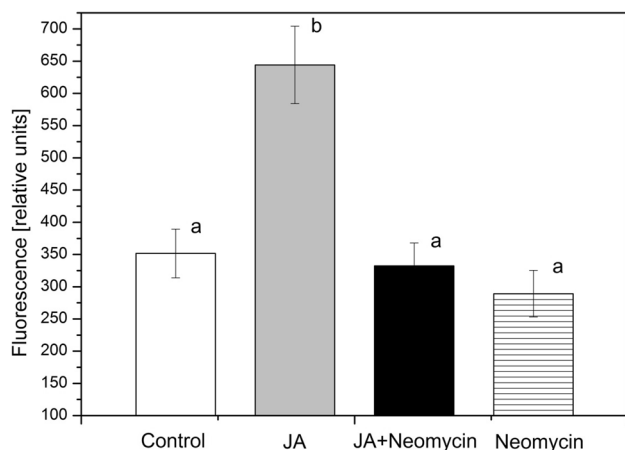
### Effect of Neomycin on Jasmonate-Induced Extrafloral Nectar Production

In unstressed *Populus trichocarpa* (poplar) plants, extrafloral nectaries release nectar within a few days only and then





**Fig. 3** Neomycin reduces JA-induced *TaPDF2.2* defense gene expression in *Triticum aestivum* (wheat) leaves. The expression levels of the *PDF2.2* gene after 10 μM JA spray (black line) and 100 μM neomycin followed by 10 μM JA spray (dotted line) were quantified by qRT-PCR at different times (hours post spray, hps). Data are represented as mean ( $\pm$  SE,  $n=4$ ); 1-way ANOVA with Fisher's LSD ( $P \leq 0.05$ ; indicated by \*\*) was used for gene expression analysis between treatments at a given time point



**Fig. 4** Neomycin inhibits the JA-induced Nepenthesin activity in *Nepenthes alata* pitcher fluid. Pitcher fluids were treated with water (white bar), 200 μM JA (final concentration) (gray bar), 100 μM neomycin (final concentration) (striped bar), and neomycin pretreatment for 6 h followed by JA addition (black bar). After one week, nepenthesin protease activity in the pitcher fluid was measured by using fluorescence substrate (PFU-093). Mean fluorescence relative unit ( $\pm$  SD,  $n=4$ ) was analyzed. Different letters indicate significant differences among treatments ( $P < 0.05$ ) in an SNK post hoc test following 1-way ANOVA

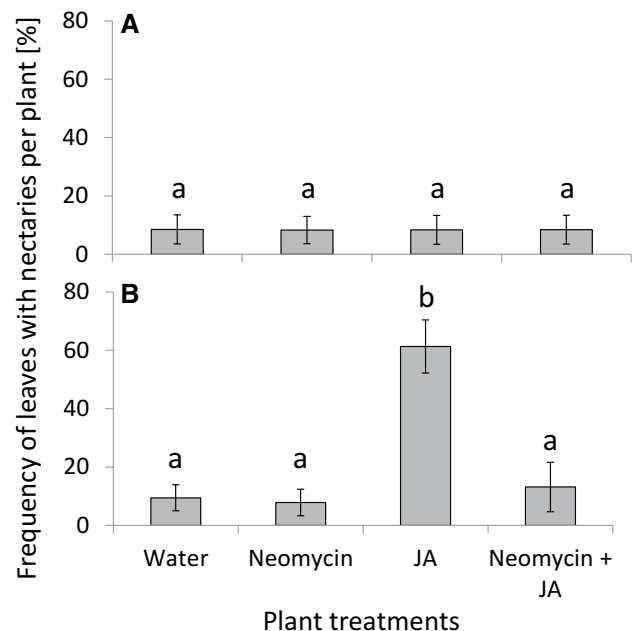
die. However, when the tree is stressed, new (secondary) nectaries are formed next to dead ones (Escalante-Pérez et al. 2012). Before the treatments, the number of leaves with extrafloral nectaries was similar among all plants and ranged from 8.3 to 18.2% per plant ( $F_{3,40}=0.004$ ,  $P=1.000$ ). Six days after treatment, we observed significant variation in

frequencies of extrafloral nectaries among the experimental plants ( $F_{3,40}=148.818$ ,  $P < 0.001$ ). Although the frequency of leaves with extrafloral nectaries formed by control plants did not change, JA treatment resulted in a significant increase in the number of functional, nectar-secreting extrafloral nectaries (Fig. 5). After JA treatment, 61.4% of all leaves were equipped with active nectaries. In contrast to the JA-only treatment, induction of plants with JA after application of neomycin did not result in significantly enhanced numbers of nectaries compared to both controls (Fig. 5). The number of leaves per plant was not affected by the different treatments ( $F_{3,40}=0.226$ ,  $P=0.878$ ).

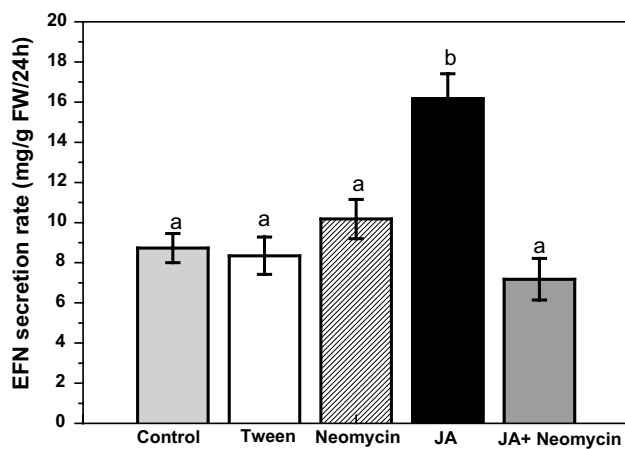
Under attack by herbivores, Lima bean (*Phaseolus lunatus*) plants produce extrafloral nectar (EFN) in order to attract ants for help, as part of their indirect defense (Kost and Heil 2008). This EFN production is also directly JA-inducible (Fig. 6) and in the presence of neomycin this jasmonate effect was significantly reduced (Fig. 6).

### Effect of Neomycin on Jasmonate-Induced Secondary Metabolites

A typical jasmonate response is the induction and accumulation of secondary metabolites (Wasternack 2007). We



**Fig. 5** Neomycin affects the jasmonic acid-induced formation of extrafloral nectaries in *Populus trichocarpa* (black cottonwood). The number of leaves per plant bearing extrafloral nectaries was counted before (A) and after (B) treatment of plants with water (control 1), neomycin (control 2), jasmonic acid (JA), and JA after an initial treatment with neomycin. Frequency of extrafloral nectaries was calculated ( $\pm$  SD,  $n=11$  plants per treatment group). Different letters indicate significant differences among treatments ( $P < 0.05$ ) in Tukey's HSD post hoc tests following 1-way ANOVA

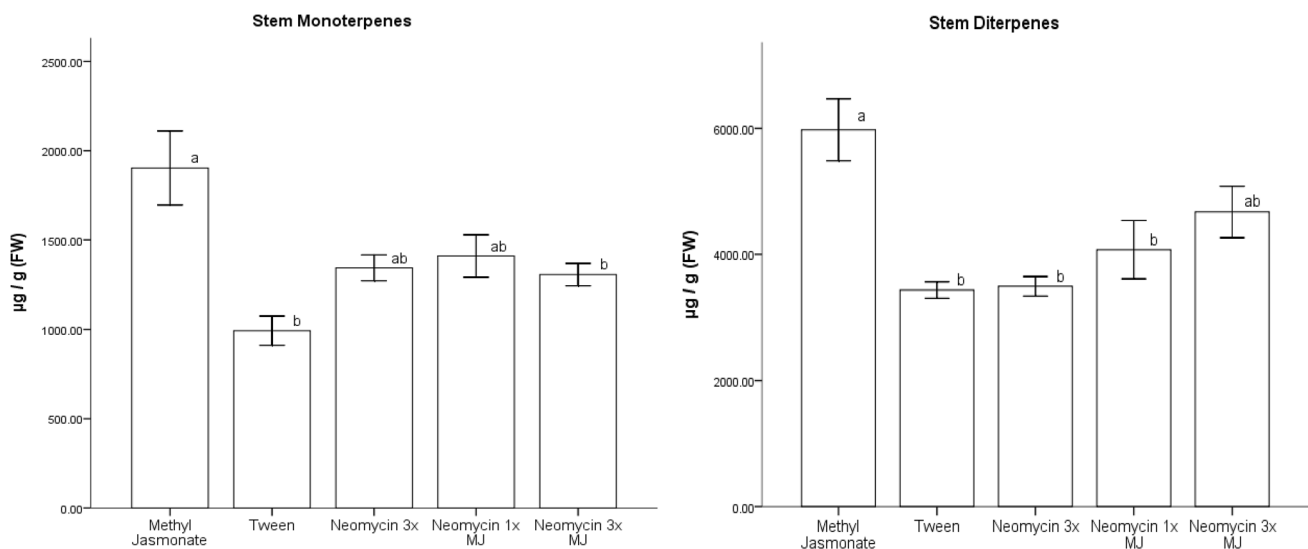


**Fig. 6** Neomycin inhibits JA-induced extrafloral nectar secretion (EFN) secretion in *Phaseolus lunatus* (Lima bean). EFN secretion rates are measured and expressed as milligrams soluble solids per gram fresh mass of leaf tissue per 24 h. Lima bean plants have been treated with water, Tween-20, JA (1 mM), neomycin (500  $\mu$ M), and neomycin (500  $\mu$ M) + JA (1 mM). Values are means ( $\pm$  SE) of 3 independent experiments ( $n=30$ ). Different letters indicate significant differences among treatments ( $P<0.05$ ) based on 1-way ANOVA utilizing SNK post hoc test

chose Norway spruce (*Picea abies*) to further investigate the neomycin effect on jasmonate-induced stem-derived mono- and diterpene accumulation of a gymnosperm. Mono- and diterpenes are the main components of the stem resin, containing the monoterpenes  $\alpha$ -pinene, camphene,  $\beta$ -pinene, myrcene,  $\delta$ -3-carene, and  $\beta$ -phellandrene as well as the

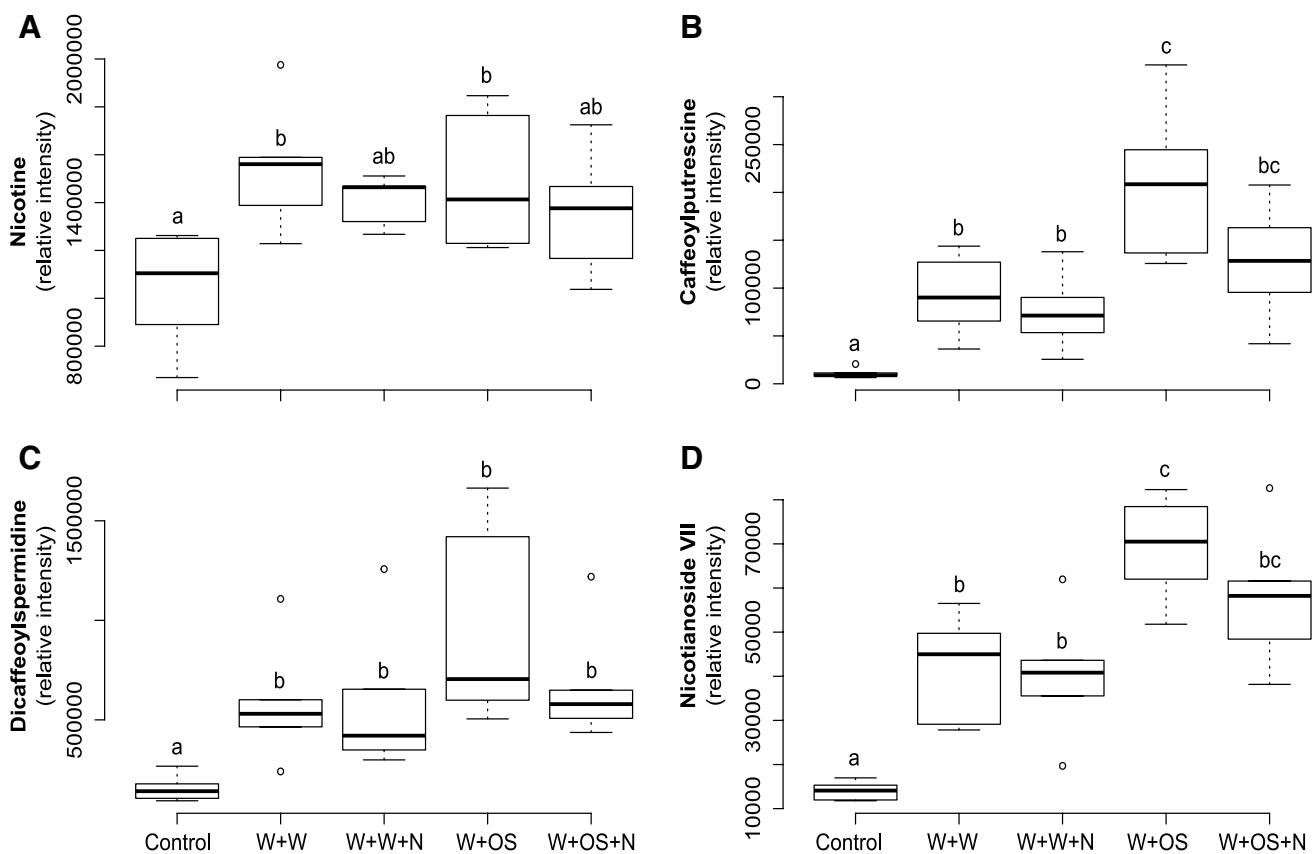
diterpenes sandracopimaric acid, isopimaric acid, levopimaric acid, dehydroabietic acid, abietic acid, and neoabietic acid (Schmidt et al. 2011). For both classes of terpenes, the treatment with neomycin together with jasmonate resulted in a significant reduction of terpene levels compared to the treatment with jasmonate alone (Fig. 7).

The wild tobacco *Nicotiana attenuata* has been developed as an ecological model plant, and its interactions with native herbivores have been extensively studied. Oral secretions (OS) from larvae of the lepidopteran specialist *Manduca sexta* elicit a specific response in *N. attenuata* plants as measured among others by changes in general and specialized metabolism; these changes are much larger than those elicited solely by wounding leaves and also include an accumulation of jasmonates which is much greater than their accumulation after wounding (Wu and Baldwin 2010). In *Arabidopsis thaliana*, it is known that neomycin selectively blocked the accumulation of *Spodoptera litura* OS-induced  $\text{Ca}^{2+}$  elevation and level of the bio-active JA-Ile. Furthermore, neomycin treatment affected the downstream expression of JA-Ile-responsive genes, *VSP2* and *LOX2*, in *Arabidopsis* (Vadassery et al. 2014). Thus, we treated the plants with *M. sexta*-derived OS in the presence and absence of neomycin, to determine whether neomycin can be used as an inhibitor of jasmonate-mediated responses to herbivory in plant–herbivore interaction studies. In the respective control, water was used instead of the OS. Four selected secondary metabolites, known to be strongly elicited by jasmonates, were analyzed: nicotine, caffeoylputrescine, dicaffeoylspermidine, and nicotianoside VII (one



**Fig. 7** Neomycin reduces the MeJA-induced terpene accumulation in *Picea abies* (Norway spruce). Clones from the same plant line were treated with MeJA (100  $\mu$ M), neomycin (100  $\mu$ M), and MeJA plus neomycin (1 $\times$  and 3 $\times$  over a period of 2 months), extracted and analyzed with by gas chromatography (GC)-MS and GC-flame ionization

detection (FID). Monoterpenes (a); diterpenes (b). Data represent the mean ( $\pm$  SE); number of replicates: samples treated with MeJA and MeJA + neo:  $n=4$ ; tween:  $n=3$ ; neomycin (3 $\times$ )  $n=2$ . Different letters designate significance ( $P\leq 0.05$ ) between treatments groups based on 1-way ANOVA utilizing Tukey's HSD post hoc analysis



**Fig. 8** The accumulation of selected jasmonate-regulated metabolites in *Nicotiana attenuata* (coyote tobacco) is reduced by neomycin treatment. Shown are nicotine (**A**), caffeoylputrescine (**B**), dicaffeoylspermidine (**C**), and one malonylated hydroxygeranyllinalool diterpene glycoside, nicotianoside VII (**D**). One single, fully expanded rosette leaf per plant (position +1) was treated on intact rosette-stage *N. attenuata* plants with wounding and water (W+W), wounding and

neomycin (100  $\mu$ M) followed 15 min later by water (W+W+N), wounding and *Manduca sexta* oral secretions (W+OS), or wounding and neomycin (100  $\mu$ M) followed 15 min later by oral secretions (W+OS+N); control leaves were left untreated ( $n=6$ ); leaves were harvested 3 days after treatment. Different letters indicate significant differences ( $P<0.05$ ) in Tukey's HSD post hoc tests following 1-way ANOVAs on log-transformed values

malonylated hydroxygeranyllinalool diterpene glycoside). For all four compounds, an induction upon OS treatment was detected, which was reduced in the presence of neomycin (Fig. 8).

## Discussion

Here, we wanted to study the potential use of the aminoglycoside neomycin as a broad tool affecting jasmonate-induced and jasmonate-dependent reactions in planta across the plant kingdom. Using eight different plant species, we were able to demonstrate the ability of neomycin to inhibit diverse types of jasmonate-induced responses, from gene induction and intracellular calcium changes to secondary metabolite and defense-related protein accumulation. It should be mentioned that we never observed any neomycin treatment-related toxicity in the plants tested. Notably, the various jasmonate responses were impaired with different efficiencies.

Neomycin affects one of the first steps of JA signaling: lowering the JA-induced cytosolic calcium increase (Fig. 1) and likely impacting the downstream signaling steps. Indeed, in the presence of neomycin jasmonate-induced nepenthesin accumulation in *Nepenthes* pitcher fluid (Fig. 4), extrafloral nectary formation in poplar (Fig. 5) and EFN production in lima bean (Fig. 6) was reduced to the control levels. The same holds true for the mRNA accumulation of wheat *TaPDF2.2* (Fig. 3), whereas *SPORAMIN* gene induction in sweet potato was diminished by about 36% (Fig. 2). In spruce, both mono- and diterpenoids induced by jasmonate treatment were reduced by more than 50% in the presence of neomycin (Fig. 7). Such differences might be due to specific assay conditions, the sensitivity of the plant tissue, a thick or thin cuticle or specifics of the signaling mechanisms of different plant species, all of which can have an impact on the effective inhibitor concentrations diffusing into the plant cells and the corresponding sensitivity of the plant. Thus, when neomycin is used in a new plant species, first a set



of experiments is recommended to find out its most efficient concentration. Interestingly, neomycin was also effective when OS derived from specialist herbivores (*M. sexta* larvae) and known to induce jasmonates in *N. attenuata*, and was used for the induction of plant responses, instead of jasmonate. Although not always significant, there was a clear trend that neomycin effectively inhibited the accumulation of several jasmonate- and herbivory-induced secondary metabolites (Fig. 8). It is known that in *A. thaliana*, neomycin affects the *S. littoralis* OS-induced accumulation of both  $[Ca^{2+}]_{cyt}$  and JA-Ile and also downstream expression of JA-Ile-responsive genes, *VSP2* and *LOX2* (Vadassery et al. 2014).

Several inhibitors of jasmonate-induced responses have already been used in plant physiological studies and thus, the question rises which are the best to use. We argue that the criteria should be high activity, chemical stability, and absence of unrelated physiological and chemical properties which would cause difficulties in interpretation. Inhibitors of enzymes that catalyze early steps in jasmonate biosynthesis such as phospholipase (inhibitor: aristolochic acid), lipoxygenases (phenidone, [1-phenyl-3-pyrazolidinone]; *n*-propyl gallate), allene oxide synthase (DIECA, [diethyl-dithiocarbamic acid]), and allene oxide cyclase (*n*-propyl gallate) (Koch et al. 1999; Engelberth et al. 2001) lack specificity. In contrast, the newly generated jarin-1 (Meesters et al. 2014) seems to be more specific and targets the enzyme catalyzing the last step of JA-Ile synthesis, while coronatine-*O*-methyloxime (Monte et al. 2014) affects the binding of JA-Ile to the co-receptor complex. Thus, by our criteria, these last two inhibitors are the best choice. Unfortunately, both compounds are not available for the majority of the broad plant science community. In contrast, neomycin is a cheap and readily available small molecule that affects signaling downstream of jasmonates, unfortunately by a still unknown molecular mechanism. This has to be investigated and unraveled in the near future and, here, it makes no sense to further speculate on any mechanism. However, the broad activity of neomycin in all tested species including mono- and dicotyledonous plants as well as a gymnosperm, qualifies this compound as a useful tool in jasmonate research. It should be noted that we attached importance to the selection of plant species which represent different taxa of higher plants. This includes *A. thaliana* but also agronomically important species such as wheat, sweet potato, poplar, spruce, and tobacco. Hence, the usage of neomycin could allow the study of jasmonate-mediated effects in plant species where up to now, genetic approaches are difficult, and mutants are not available.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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