J Plant Growth Regul (2011) 30:504–511
DOI 10.1007/s00344-011-9212-1

A Whole-Plant Microtiter Plate Assay for Drought Stress Tolerance-Inducing Effects

Torsten Geissler · Ludger A. Wessjohann

Received: 28 January 2011 / Accepted: 26 April 2011 / Published online: 21 May 2011

Abstract The frequency and intensity of extreme weather events and global temperature are rising, which poses a potential threat to life, specifically crops, and therefore food and bioenergy supply. Reduced water availability has the most severe impact on potential grain yield. Negative effects of transient drought stress (dry spells) can be countered by drought tolerance-inducing chemicals. In search for useful compounds, biochemical assays are fast but limited in scope, whereas whole-plant assays are slow, require large amounts of compounds, and are usually not concentration-related. Here we report the development of a fast, concentration-dependent whole-plant assay using the fast growing duckweed *Lemna minor* L. 4-Amino-1,8-naphthalimide (**1**) and the imidacloprid metabolite 6-chloronicotinic acid (**2**) were affirmed as drought stress tolerance enhancers. Both also reduce oxidative stress-induced cell death in *Arabidopsis thaliana* (L.) Heynh. cell suspension culture but show differences in their mode of action.

Keywords *Lemna minor* · Automated plant phenotyping · PARP · ARTD · Water stress · Abiotic stress · Imidacloprid

Dedicated to Professor Dr. Hans-Joachim Niclas on the occasion of his 70th birthday.

T. Geissler · L. A. Wessjohann (✉)
Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany
e-mail: wessjohann@ipb-halle.de

Introduction

Plants periodically encounter environmental stresses that negatively affect growth, development, and productivity. During the last decades, breeders substantially improved the yield of many crop species. This often is less associated with increased yield potential or heterosis than with increased stress tolerance (Tollenaar and Lee 2002). The potential of plant productivity becomes apparent when comparing the average yield of plants with the record yield, representing almost ideal conditions for the plant. In this context, typical yield losses can amount to between 60% and 80%, depending on the crop plant (Bray and others 2002). Although much of the plant and agrochemical research today is focused on biotic stress, nearly 90% of all crop loss is caused by abiotic stress factors like freezing, elevated temperature, UV radiation, and drought. Among these, the most serious one is inadequate water supply or drought stress (DS). For example, in soybean DS alone causes a yield loss of 20–46% (Zhang and others 2007). Therefore, plant breeders, biotechnologists, and agrochemists endeavor to increase the tolerance of crop plants against DS, especially with the use of agrochemicals as plant growth regulators. For ornamental plants, an accompanying greening effect of DS tolerance-enhancers defines an additional market.

In the last years, very few substances showing drought stress tolerance-inducing effects (DSTIE) on plants have been identified. The most prominent example is imidacloprid (Bayer AG), which decomposes to compounds that have DSTIE (Thielert 2006). This observation may have contributed to the identification of poly-(ADP-ribose)-polymerases (PARP). PARPs, also known as *Diphtheria* toxin-like ADP-ribosyltransferases, have been validated as key target proteins for the improvement of drought

tolerance in plants. PARPs catalyze the post-translational modification of mainly nuclear target proteins using NAD^+ as substrate. PARP is well investigated in animal models, and it is assumed that oxidative stress signaling leads to an over-activation of the enzyme, resulting in a decreased intracellular NAD^+ pool. This causes necrotic cell death (David and others 2009). In plants, a reduction of the activity of PARPs by expression of RNAi constructs was found to increase plant tolerance of various abiotic stresses such as heat stress, high light stress, and DS. Presumably, PARPs play a crucial role in energy metabolism and it is assumed that by maintenance of energy homeostasis, stress tolerance is conferred (De Block and others 2005). Furthermore, it was shown that plants expressing the RNAi construct exhibit higher growth rates under stress conditions but also lower growth rates under well-watered conditions compared to control plants (Jansen and others 2009). In conclusion, these results demonstrate that a permanent genetic knockdown of PARP is less desirable than the temporary inhibition of PARP.

Various fungicides of the triazole family (for example, paclobutrazol, uniconazole, triadimephon) induce a general stress tolerance when they are applied as a soil drench or seed treatment (Fletcher and Hofstra 1988). However, treated plants show retarded growth because the triazoles also inhibit gibberellin biosynthesis (Fletcher and Hofstra 1988). More recently it was found that salicylic acid and derivatives thereof provide tolerance against a variety of abiotic stress factors (Senaratna and others 2000). Nevertheless, salicylic acid is also involved in the induction of a hypersensitive response *in planta* (Crozier and others 2010). The induction of programmed cell death with acetyl salicylic acid was demonstrated (Garcia-Heredia and others 2008). Both properties are unfavorable for the development as agrochemicals.

Therefore, it is very important to detect substances that feature higher efficiency and selectivity in increasing the capacity of plants to withstand DS conditions. For lead structure identification and optimization, robust assays are needed that use fast-growing plants and low amounts of compounds and are concentration-dependent. To our knowledge there is no assay reported that features all of these requirements. On the one hand, large plants are used that grow slowly and have to be sprayed with considerable amounts (several milligrams) of compounds and allow no direct concentration-dependent evaluation (Mascher and others 2005; Walter and others 2007; Jansen and others 2009). On the other hand, smaller systems such as leaf discs have a short lifetime and show a permanent wounding situation, therefore making water loss or vitality difficult to normalize (Saradadevi and others 1996). Biochemical assays need much smaller amounts of compounds and are concentration-dependent, but there is both a lack of validated target proteins and low relevance for plant application. In this report we describe the development of a bioassay for substances with DSTIE using the fast-growing duckweed *Lemna minor* L. as the test organism. Because *Lemna* plants propagate as genetically identical clones producing a flat leaf surface suitable for 2D-imaging readouts, the assay can be run in a medium-throughput format.

Materials and Methods

Lemna Growth Assay

Axenic cultures of *L. minor* (duckweed) were obtained from the Canadian Phycological Culture Centre (strain number CPCC 490). Plants were precultured according to the standardized method of the "Duckweed growth inhibition test" (ISO/DIS 20079, 2003) in Steinberg medium. Reduced water potential medium was prepared by adding PEG 6000 under sterile conditions to Steinberg medium (for −0.2 MPa: 180.0 g/l and for −0.3 MPa: 211.9 g/l) according to Michel and Kaufmann (1973), followed by filtration using a 0.45-µm polyvinylidene fluoride filter.

Plants were grown under constant conditions in a phytochamber for 7 days under continuous light (ca. 100 µmol m^{-2} s^{-1}) at 24°C (70% humidity) in 24-well plates, each well containing 1,980 µl Steinberg medium. Tested compounds were added from 10 mM stock solutions [solvents: DMSO or 50 mM phosphate buffer (pH 5.5)]. To achieve a constant stock solvent concentration of 0.1% per well, additional DMSO or phosphate buffer (pH 5.5) was added where necessary, that is, for low test concentrations of compounds. DMSO itself did not show any effect at 0.1% (v/v). Afterward, a three- to four-fronded colony of *L. minor* was transferred to each well of the microplate. To assess the growth rate, high-resolution pictures of the plates were taken every 24 h using the Lemnatec Scanalyser PL equipped with SAW Duckweed Software LT-0004. Due to natural variation, the initial projected frond area A_{p0} at assay start (t_0) cannot be held constant. Therefore, the averaged logarithmic growth rate μ was used as a growth parameter, which was determined by linear regression analysis (Eq. 1). Data represent the mean of three independent experiments with each consisting of four replicates.

$$\ln(A_p) = \mu \cdot t + \ln(A_{p_0}) \quad (1)$$

To check the ideal plate size, standard 6-, 12-, 24-, and 48-well plates and a 400 ml beaker as ISO standard were compared (data not shown). A too small well size and

nutrient volume might affect results by too little nutrient content or by surface area limitations at the final stages of plant growth. The smallest volume/surface without limiting growth at 0.2 MPa or more drought-stressed *L. minor* plants was found in 24-well plates. For the experiments using the highest nutrient volume, 300 ml of Steinberg medium containing the appropriate amounts of PEG 6000 was transferred to 400 ml beakers. After transferring colonies of 9–12 fronds in total per replicate, compounds of interest and/or solvents were added. After incubation for 7 days, growth rates were determined as described above. In the following, only 24-well plate and 300 ml ISO-volume results are discussed as lower and upper limits of the assay conditions tested.

Plant Cell Assay

Wild-type Col-0 *Arabidopsis thaliana* (L.) Heynh. cells were obtained from the Department of Stress and Developmental Biology, Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany. Suspension cultures were grown in 250 ml flasks in Murashige and Skoog medium supplemented with Gamborg B5 vitamins and 1 mg/ml 2,4-dichlorophenoxyacetic acid (MS + medium) at 25°C in the dark. Culture maintenance was achieved by transferring 4 ml of a 7-day-old culture into 40 ml fresh medium. For the experiments, 1 ml of freshly subcultured cell suspension was transferred to each cavity of a 12-well plate.

To induce oxidative stress, 15.5 µl of 30% hydrogen peroxide solution was added. Tested compounds were added from 10 mM stock solutions (solvents: DMSO or MS + medium). To achieve a constant concentration of stock solvent of 1% per well, additional DMSO or MS + medium was added where necessary, that is, for low concentrations of test compounds. The well was filled with MS + medium to reach a total volume of 2 ml. Controls were prepared in a similar manner.

For determination of cell death, the Evans blue method was used (Levine and others 1994). The cell cultures were incubated for 15 min with 0.05% Evans blue and subsequently washed with sterile bidestilled H_2O to remove unbound dye. Evans blue bound to dead cells was extracted using 50% methanol with 1% SDS for 30 min at 50°C. The absorbance was measured on an Eppendorf biophotometer at 595 nm. Data represent means of three replicates.

PARP Assay

PARP activity was determined using the HT Universal PARP Assay (Trevigen) with the human PARP-1 (ARTD1) enzyme according to the manufacturer's protocol. Absorbance of colorimetric substrates was measured on a Molecular Devices Spectramax M5 microplate reader.

Statistics

Normal distribution of the results was tested using the D'Agostino-Pearson omnibus K2 test, and statistical significance was determined using Student's *t*-test. For statistical analysis the software GraphPad Prism 5.0 was used.

Results and Discussion

There is no suitable assay described in the literature that allows a fast, concentration-dependent screening of compound libraries on whole plants, in general and specifically also not for drought stress tolerance (DST) inducers. Therefore, an assay was developed that uses the fast-growing duckweed *L. minor*, allowing high throughput on a whole-organism basis. This model plant has been employed previously in ecotoxicology screening for the "Duckweed growth inhibition test" described in ISO/DIS 20079 (2003). To simulate DS, media with reduced water potential were used. During the screening process, 4-amino-1,8-naphthalimide (**1**), an inhibitor of human PARP-1, and 6-chloronicotinc acid (**2**), an imidacloprid degradation product, were identified to exhibit DSTIE (Fig. 1).

In the developed microplate assay, compound **1** significantly increases the growth rate of *Lemna* plants under DS conditions (−0.3 MPa) at 10 and 1 µM by 16.3 and 12.6%, respectively, relative to untreated stressed control plants (Fig. 2a). At high concentrations of **1** (100 µM) the growth rate drops down to the control level, which might be attributed to secondary effects of compound **1**. The desired effect is observable only under stress but not under normal growing conditions (Fig. 2b). To confirm the results, a similar assay using 300 ml nutrient volume was performed, which is more similar to the traditional ecotoxicology assay described in the ISO/DIS 20079 (2003). Here equal results were obtained, whereas compound **1** increases the growth rate of *Lemna* at 10 and 1 µM only under DS conditions but not under well-watered conditions (Fig. 3).

Compound **2** also exhibits activity in the developed *Lemna* microplate assay, but only at −0.2 MPa, increasing the growth rate by 13.6% at 100 µM (Fig. 4a). Higher concentrations of **2** (>100 µM) cause chlorosis, therefore, no effects could be observed at −0.3 MPa (data not shown). Even under well-watered conditions, minor growth-promoting effects of **2** could be observed (Fig. 4b). Confirmation of the results from the 24-well (2 ml) microplate assay was also obtained for 300 ml nutrient volume, wherein compound **2** increases the growth rate at 100 µM by approximately 10% (Fig. 5a). Under well-watered conditions in either assay, no significant growth

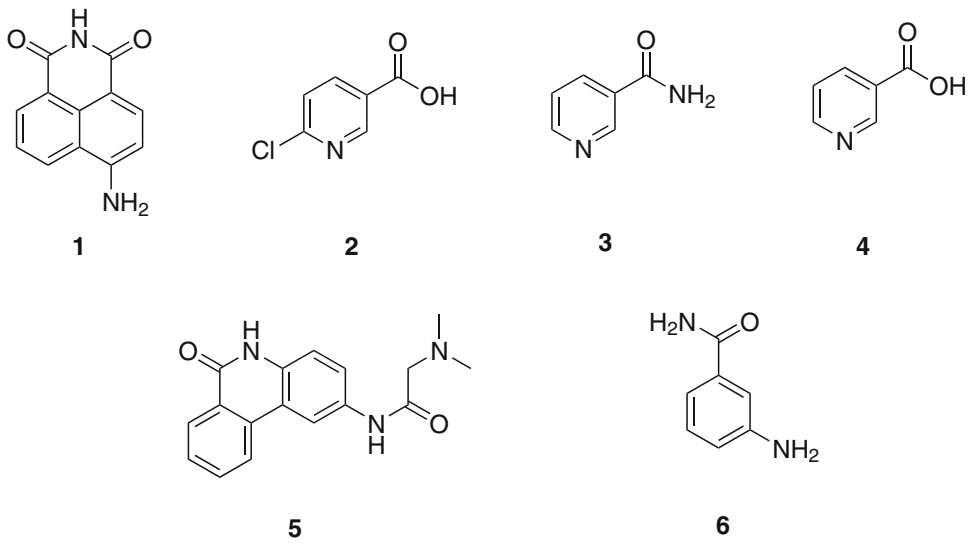

Fig. 1 4-Amino-1,8-naphthalimide (**1**), 6-chloronicotinic acid (**2**), nicotinamide (**3**), nicotinic acid (**4**), PJ-34 (**5**), and 3-aminobenzamide (**6**)

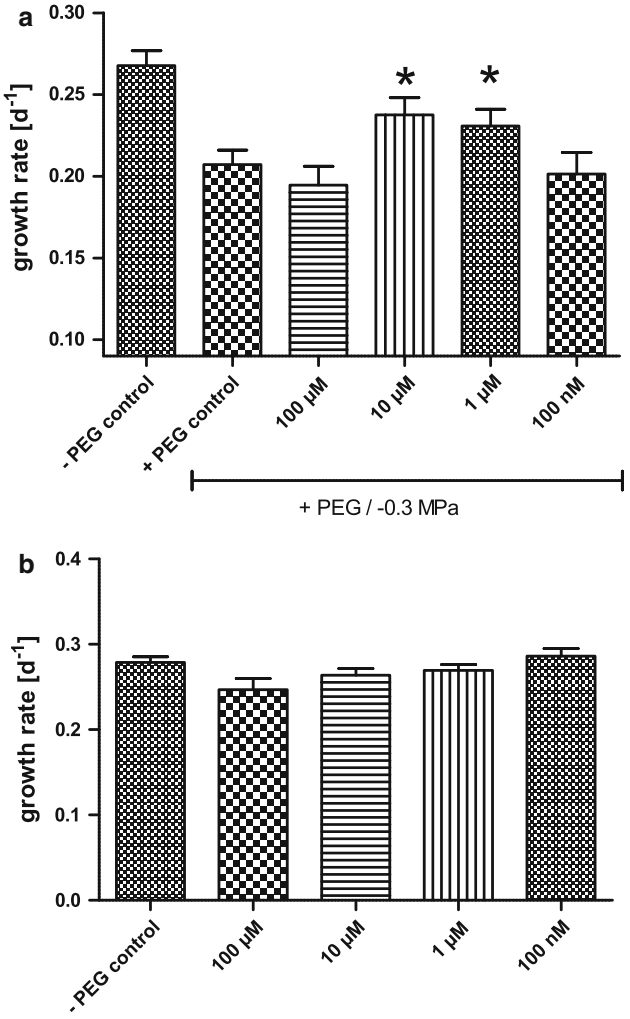

Fig. 2 Growth rates of *Lemna* plants grown in 24-well microplates under **a** drought stress (−0.3 MPa) and **b** well-watered conditions treated with different concentrations of 4-amino-1,8-naphthalimide (**1**). Data are mean ± SEM; *Asterisk* represents significant differences compared to untreated stressed control with $P < 0.05$

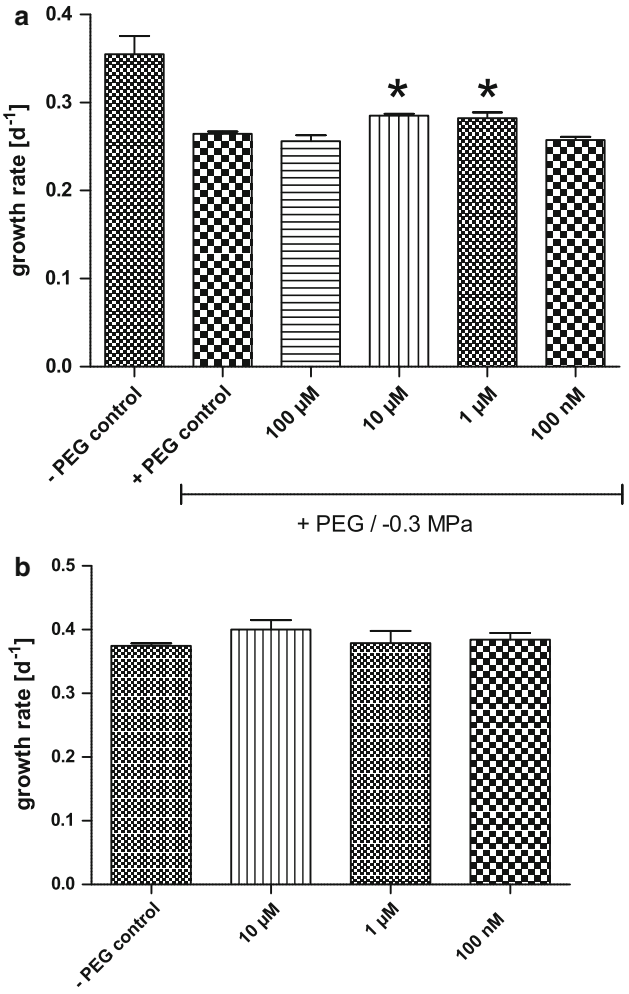

Fig. 3 Growth rates of *Lemna* plants under **a** drought stress (−0.3 MPa) and **b** well-watered conditions treated with different concentrations of 4-amino-1,8-naphthalimide (**1**) cultivated in 300 ml nutrient medium. Data are mean ± SEM; *Asterisk* represents significant differences compared to untreated stressed control with $P < 0.05$

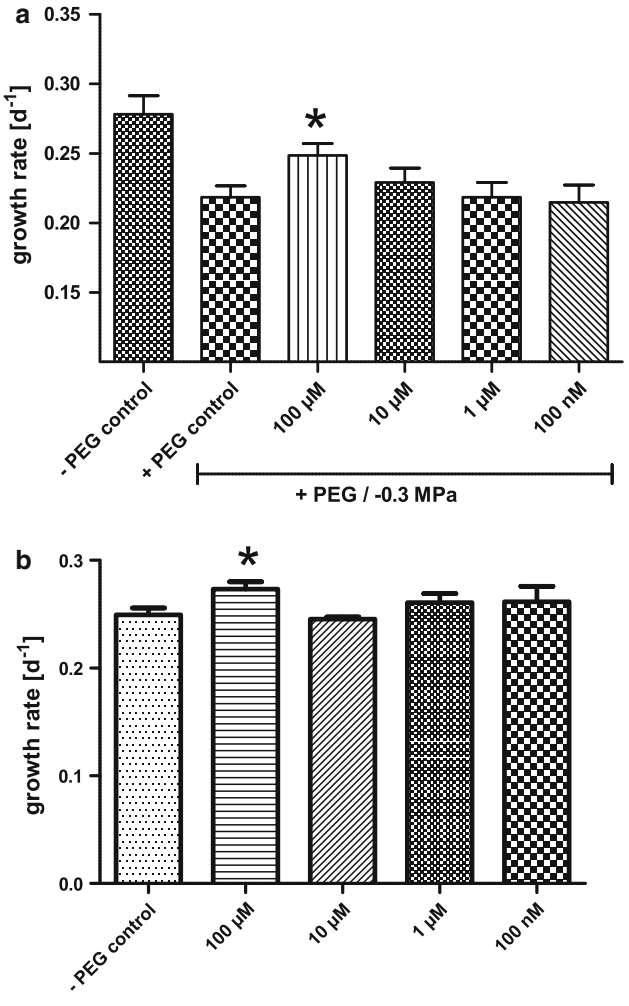

Fig. 4 Growth rates of *Lemna* plants grown in 24-well microplates under **a** drought stress (−0.2 MPa) and **b** well-watered conditions treated with different concentrations of 6-chloronicotinic acid (**2**). Data are mean ± SEM; *Asterisk* represents significant differences compared to untreated stressed control with $P < 0.05$

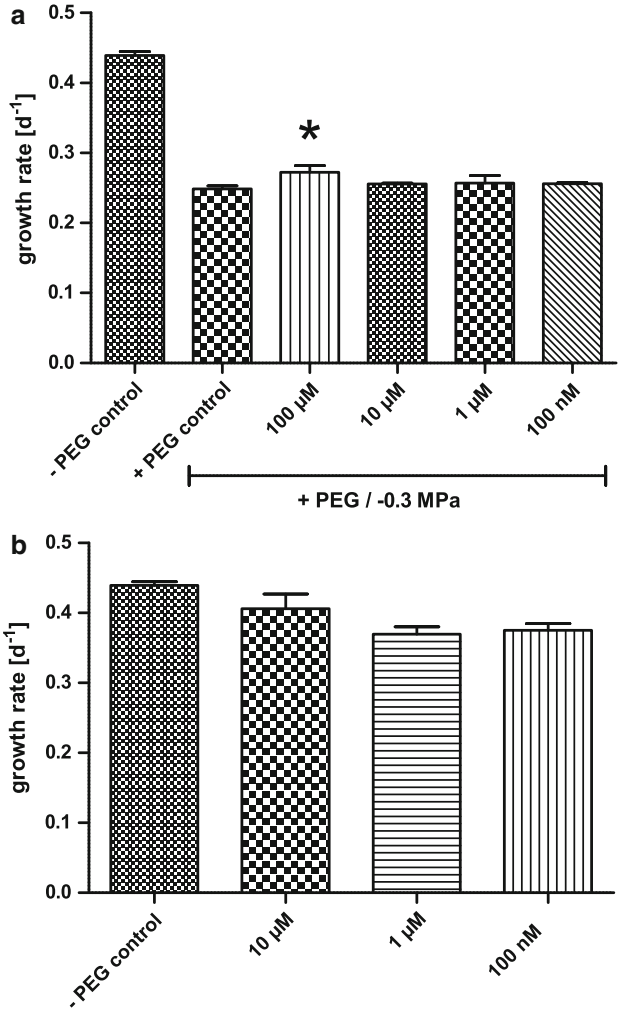

Fig. 5 Growth rates of *Lemna* plants under **a** drought stress (−0.2 MPa) and **b** well-watered conditions treated with different concentrations of 6-chloronicotinic acid (**2**) cultivated in 300 ml nutrient medium. Data are mean ± SEM; *Asterisk* represents significant differences compared to untreated stressed control with $P < 0.05$

differences between treated and untreated plants could be observed (Figs. 4b, 5b).

Primary environmental stress factors can be converted intracellularly into secondary oxidative stress, leading, for instance, to membrane oxidation or DNA strand breaks (Smirnoff 1993). Therefore, cell-based oxidative stress experiments were chosen to verify the obtained *Lemna* results using *A. thaliana* cells. Cells were treated with 75 mM H_2O_2 in the presence or absence of compounds **1** and **2**. Both compounds significantly reduce oxidative stress-induced cell death at 10 µM (**1** −20%; **2** −38%; Fig. 6). The reason for the higher activity compared to the *Lemna* assay might be that there is a better bioavailability in cellular systems or their reduced capacity for metabolic degradation. Besides, both compounds do not exhibit effects on the cells in control experiments. The results match those obtained from the *Lemna* assay and therefore give rise to the assumption that our developed screening assay can be used for screening substances with DSTIE and that its results can be transferred to systems using higher organisms.

Compound **1** is described as an inhibitor of the human PARP-1 (Banasik and others 1992). Therefore, **1** and **2** were tested for their inhibitory effects on the PARP-1 enzyme using the HT Universal PARP Assay (Trevigen). As controls, the known inhibitors PJ-34 (**5**) (Abdelkarim and others 2001) and 3-aminobenzamide (**6**) (Banasik and others 1992) were used. Surprisingly, only the naphthalimide **1** exhibits remarkable inhibitory activity against PARP-1 ($IC_{50} = 2.8$ µM). In contrast, chloronicotinate **2** does not inhibit the enzyme, even at concentrations up to 2 mM (Fig. 7).

Compound **2** might mimic nicotinic acid by increasing the intracellular nicotinic acid(s) pool: more NAD^+ can be

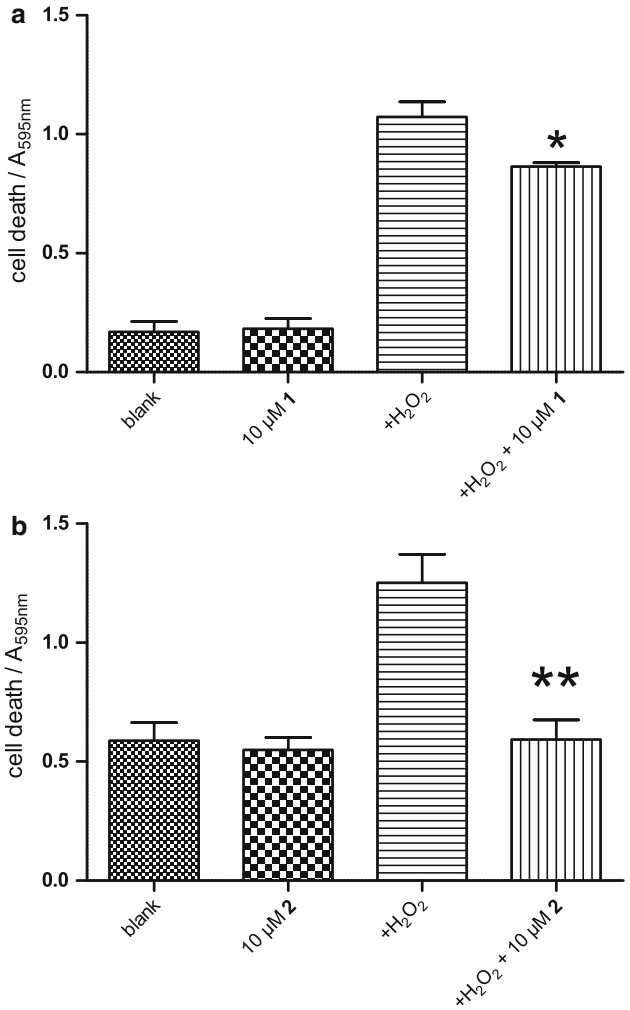

Fig. 6 Oxidative stress-induced cell death of *A. thaliana* suspension culture cells caused by H_2O_2 in the presence or absence of 4-amino-1,8-naphthalimide (**1**; **a**) or 6-chloronicotinate (**2**; **b**). Data are mean ± SEM; *Asterisk* represents significant differences with $P < 0.05$

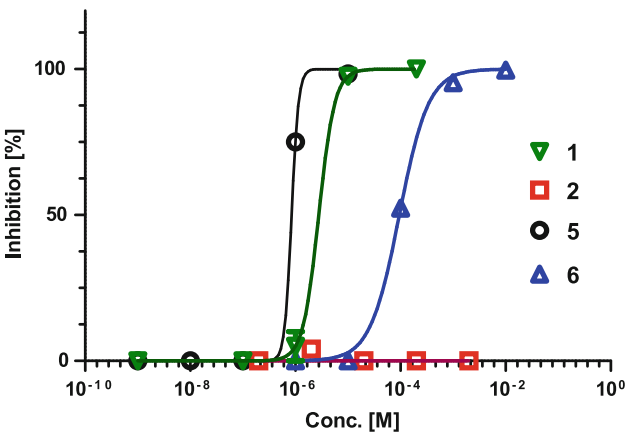

Fig. 7 PARP inhibition dose–response curves of compounds **1** and **2** and reference inhibitors as controls (**5**, **6**). Data are mean ± SEM

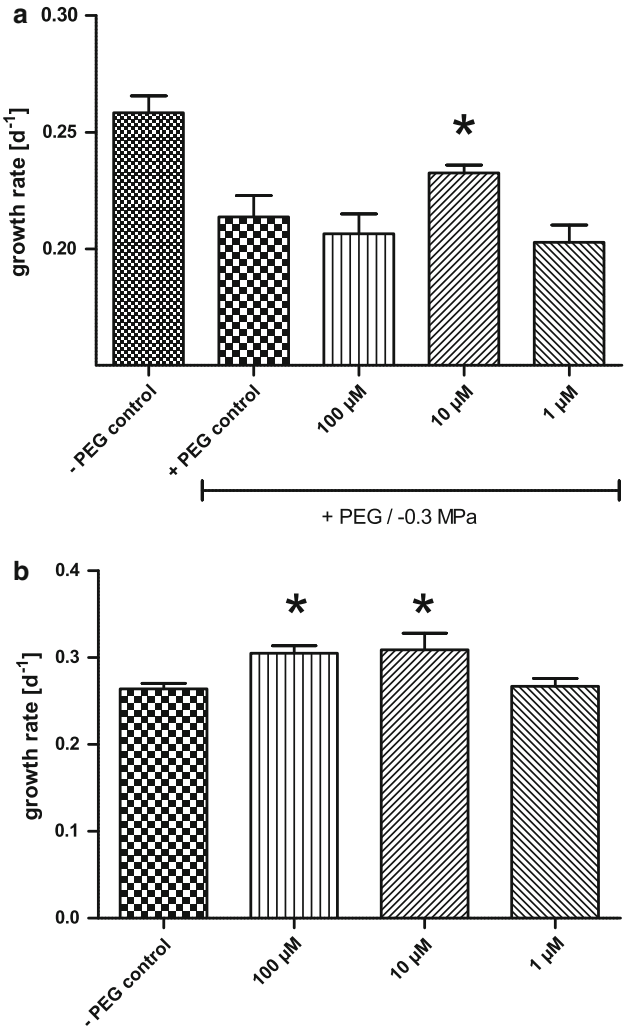

Fig. 8 Growth rates of *L. minor* plants grown in 24-well microplates under **a** drought stress (−0.3 MPa) and **b** well-watered conditions treated with different concentrations of nicotinate (**4**). Data are mean ± SEM; *Asterisk* represents significant differences compared to untreated stressed control with $P < 0.05$

synthesized as NAD^+ pools are reduced by PARP activity. This would correspond to a vitaminic effect, and therefore **2** would be needed in stoichiometric amounts. This can explain the high concentration of **2** required for positive effects in our DSTIE assays. To prove this assumption, nicotinamide (**3**) and nicotinic acid (**4**; vitamin B3) were also tested, as they should also exhibit DSTIE in this case. Indeed, **3** and **4** enhance the growth rate of *L. minor* plants under DS conditions, even at lower concentrations than the unnatural 6-chloro derivative **2** (Figs. 8a, 9a). Furthermore, nicotinate (**4**) and nicotinamide are able to increase growth rates under well-watered conditions (Fig. 8a). This shows that the effect of **3** and **4** under water stress conditions should not be accepted as proof of an influence on water-deficit tolerance. These findings support the hypothesis that the chloronicotinate **2** may increase the intracellular

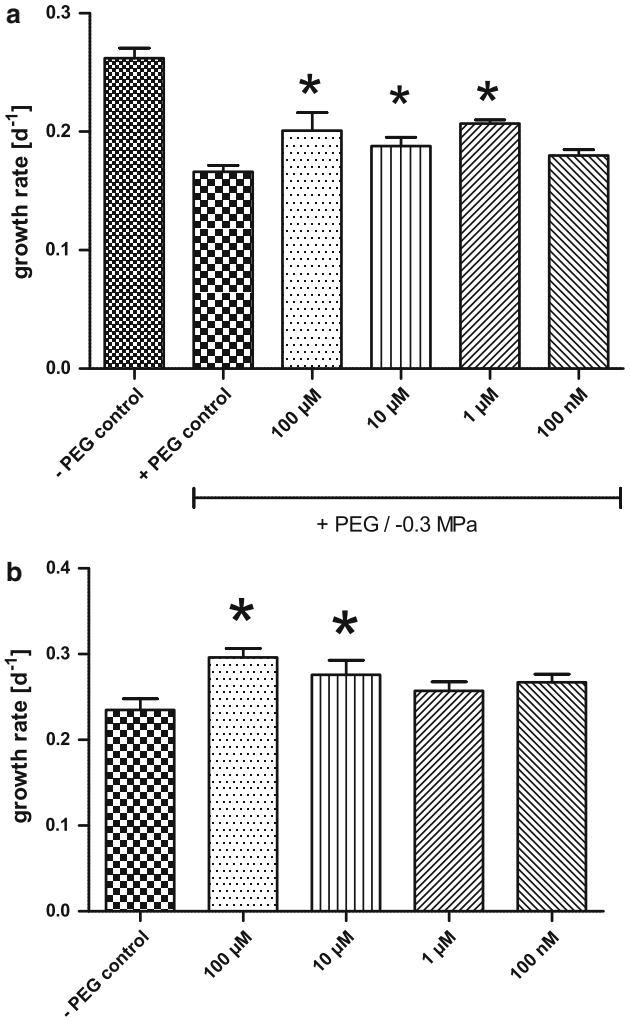

Fig. 9 Growth rates of *L. minor* plants grown in 24-well microplates under **a** drought stress (−0.3 MPa) and **b** well-watered conditions treated with different concentrations of nicotinamide (**3**). Data are mean ± SEM; *Asterisk* represents significant differences compared to untreated stressed control with $P < 0.05$

nicotinic acid (like) pool of the plant and that it may act as an effector on the primary metabolism by its vitaminic or nutrient effects. This would also explain the high concentrations required for the effects.

Imidacloprid, an insecticide of the chloronicotinyl class, also improves survival and growth rate of drought-stressed *A. thaliana* and barley plants in disease- and pest-free studies (Thielert 2006). Interestingly, the chloronicotinate **2** is one of the major degradation products of this pesticide. According to the manufacturer's protocol, imidacloprid (Confidor®) is applied as a 0.035% solution (∼95 µM). Because in our assays similar concentrations showed an activity as nicotinate supplement and not as PARP inhibitor, we suggest that the DSTIE of imidacloprid likely is attributed to an increase of the nicotinic acid pool or to an interaction with other proteins.

In summary, 4-amino-1,8-naphthalimide (**1**) and the 6-chloronicotinic acid derivative **2** are responsible for increasing drought stress tolerance in a newly developed whole-plant *Lemna* assay. Only **1** seems to act as an inhibitor of the sole validated DSTIE target enzyme PARP.

Acknowledgments Sylvia Krüger is kindly acknowledged for her technical support. This work was supported by the state of Saxony-Anhalt (KM-LSA) through the Agrochemical Institute Piesteritz (AIP).

References

Abdelkarim GE, Gertz K, Harms C, Katchanov J, Dirnagl U, Szabo C, Endres M (2001) Protective effects of PJ34, a novel, potent inhibitor of poly(ADP-ribose) polymerase (PARP) in in vitro and in vivo models of stroke. Int J Mol Med 7:255–260

Banasik M, Komura H, Shimoyama M, Ueda K (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. J Biol Chem 267:1569–1575

Bray EA, Bailey-Serres J, Weretilnyk E (2002) Response to abiotic stresses. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry & molecular biology of plants. Wiley, New York, pp 1158–1203

Crozier A, Kamiya Y, Bishop G, Yokota T (2010) Biosynthesis of hormones and elicitor molecules. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, MD, pp 850–929

David KK, Andrabi SA, Dawson TM, Dawson VL (2009) Parthanatos, a messenger of death. Front Biosci 14:1116–1128

De Block M, Verduyn C, De Brouwer D, Cornelissen M (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. Plant J 41:95–106

Fletcher RA, Hofstra G (1988) Triazoles as potential plant protectants. In: Berg D, Plembel M (eds) Sterol biosynthesis inhibitors: pharmaceutical and agrochemical aspects. VCH Publishers, Weinheim, pp 321–331

Garcia-Heredia JM, Hervas M, De la Rosa MA, Navarro JA (2008) Acetylsalicylic acid induces programmed cell death in *Arabidopsis* cell cultures. Planta 228:89–97

ISO/DIS 20079 (2003) Water quality—determination of the toxic effect of water constituents and waste water to duckweed (*Lemna minor*)—Duckweed growth inhibition test, ISO TC 147/SC 5

Jansen M, Gilmer F, Biskup B, Nagel KA, Rascher U, Fischbach A, Briem S, Dreissen G, Tittmann S, Braun S, De Jaeger I, Metzlaff M, Schurr U, Scharr H, Walter A (2009) Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in *Arabidopsis thaliana* and other rosette plants. Funct Plant Biol 36:902–914

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583–593

Mascher R, Nagy E, Lippmann B, Hörnlein S, Fischer S, Scheiding W, Neagoe A, Bergmann H (2005) Improvement of tolerance to paraquat and drought in barley (*Hordeum vulgare* L.) by exogenous 2-aminoethanol: effects on superoxide dismutase activity and chloroplast ultrastructure. Plant Sci 168:691–698

Michel BE, Kaufmann MR (1973) Osmotic potential of polyethyleneglycol 6000. Plant Physiol 51:914–916

Saradadevi K, Padmasree K, Raghavendra AS (1996) Correlation between the inhibition of photosynthesis and the decrease in area

of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea (*Pisum sativum*). Physiol Plant 96:395–400

Senaratna T, Touchell D, Bunn E, Dixon K (2000) Acetyl salicylic acid (Aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regul 30:157–161

Smirnoff N (1993) Tansley review no. 52. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125:27–58

Thielert W (2006) A unique product: the story of the imidacloprid stress shield. Bayer CropScience (formerly Pflanzenschutz-Nachrichten Bayer) 59:73–86

Tollenaar M, Lee EA (2002) Yield potential, yield stability and stress tolerance in maize. Field Crops Res 75:161–169

Walter A, Scharr H, Gilmer F, Zierer R, Nagel KA, Ernst M, Wiese A, Virnich O, Christ MM, Uhlig B, Junger S, Schurr U (2007) Dynamics of seedling growth acclimation towards altered light conditions can be quantified via GROWSCREEN: a setup and procedure designed for rapid optical phenotyping of different plant species. New Phytol 174:447–455

Zhang M, Duan L, Tian X, He Z, Li J, Wang B, Li Z (2007) Uniconazole-induced tolerance of soybean to water deficit stress in relation to changes in photosynthesis, hormones and antioxidant system. J Plant Physiol 164:709–717