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UV-B- and oxidative stress-induced increase in nicotinamide and trigonelline and inhibition of defensive metabolism induction by poly(ADP-ribose)polymerase inhibitor in plant tissue

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Abstract Nicotinamide and trigonelline contents increased in *Catharanthus roseus* tissue culture after exposure to 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) or vanadylsulfate and in *Pisum sativum* leaves after exposure to UV-B radiation. Vanadylsulfate increased phenylalanine ammonia-lyase (PAL) activity and the content of reduced and oxidized glutathione in *C. roseus* tissue culture. The increases in PAL activity caused by 2 mM AAPH or 0.2 mM vanadylsulfate were prevented by 0.1 mM 3-aminobenzamide (3-AB), an inhibitor of poly(ADP-ribose)polymerase. Present results support the hypothesis [Berglund T., FEBS Lett. (1994) 351, 145–149] that nicotinamide and/or its metabolites may function as signal transmittors in the response to oxidative stress in plants and that poly(ADP-ribose)polymerase has a function in the induction of defensive metabolism.

Key words: Nicotinamide; Oxidative stress; Poly(ADP-ribose)polymerase; Trigonelline; UV-B

1. Introduction

The mechanisms of the early stress response prior to the expression of defense-related genes in plant cells are largely unknown. Certain non-protein compounds in plants are considered to have stress signal functions, e.g. jasmonic acid, salicylic acid, abscisic acid and ethylene [1–3]. The level of action of these compounds (local, systemic or both) is still under debate. The induction of defensive metabolism is often associated with increased tissue levels of reactive oxygen species (ROS) and increased cellular levels of free cytosolic calcium [4]. The increase in ROS is probably an important part in the process of defense induction and can also constitute a defense function per se [4,5].

The location of the primary interaction between a stressor and the cell may differ considerably depending on the nature of the stressor. Thus, the primary interaction can, for example, occur at the level of the plasma membrane, in the cytoplasm including its organelles, or in the DNA of the nucleus or the organelles. Among the environmental stressors reacting close to the plasma membrane is probably ozone. UV-B radiation may, depending on the wavelength and dose, reach different compartments of the cell and cause various types of effects, such as DNA damage [6], especially thymine dimerization, and oxidative damage, such as lipid peroxidation [7,8]. Certain compounds will interact primarily with DNA and cause damage at this level. Depending on the nature of the compound or physical agent and its site of interaction, there may be various sensor functions at different loci in the cell. It is also a possible that the relative importance of these sensor functions may change with the state of differentiation and age of the cell/tissue.

It has been hypothesized that nicotinamide (NIC) and/or its metabolites nicotinic acid and *N*-methyl nicotinic acid (trigonelline; TRIG) may constitute a link between various types of stresses, especially stress causing DNA strand breaks, and the induction of defensive metabolism in eukaryotic cells [9–12]. An important source of NIC release in response to stress is the DNA strand break-activated eukaryotic enzyme poly(ADP-ribose)polymerase (PADPRP) [13,14]. PADPRP is present in nuclei of various plant species [15] and shows similarities to animal PADPRP [16]. In plants, NIC can be metabolized to N-glucose nicotinic acid or TRIG via nicotinic acid [17]. The first step in the pathway from NIC to TRIG is catalyzed by a nicotinamide amidohydrolase, forming nicotinic acid, and the second step, from nicotinic acid to TRIG, is catalyzed by an S-adenosylmethionine (SAM)-dependent *N*-methyltransferase [17].

Many plant stressors, biotic as well as abiotic, are associated with oxidative stress and increased levels of reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide ions (O_2^-) and hydroxyl radicals ($^{\cdot}OH$) [5,7]. Exposure of plant tissue to UV-B light [8,18,19] or 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) [20] is associated with increased antioxidative defense metabolism. Both UV-B [8] and AAPH [20] exposure are known to cause increased lipid peroxidation in plant tissue. Oxovanadium compounds cause secondary metabolite accumulation and activation of antioxidative metabolism in plant tissue [21,22]. From studies with pure chemicals it is known that vanadyl ions can increase the level of ROS [23]. Here we show that $VOSO_4$ induces increased levels of reduced and oxidized glutathione and increased activity of phenylalanine ammonia-lyase (PAL) in plant tissue and we use $VOSO_4$ as a stressor in connection with the PADPRP-inhibitor 3-AB. The present results also show that exposure of intact plants to UV-B and exposure of plant tissue cultures to AAPH or vanadylsulfate ($VOSO_4$) also cause a rise in the tissue levels of NIC and TRIG.

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Abbreviations: 3-AB, 3-aminobenzamide; 3-MB, 3-methoxybenzamide; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; CHS, chalcone synthase; GSSG, oxidized glutathione; GSH, reduced glutathione; GSH_{tot}, total glutathione; NIC, nicotinamide; PADPRP, poly(ADP-ribose)polymerase; PAL, phenylalanine ammonia-lyase; ROS, reactive oxygen species; TRIG, trigonelline; $VOSO_4$, vanadylsulfate

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NIC increases the levels of mRNAs for defense-involved genes, such as those encoding chalcone synthase (CHS) and glutathione reductase (GR) in *Pisum sativum* tissue cultures [10], as well as increased accumulation of anthocyanin in *Catharanthus roseus* tissue culture [9]. NIC treatment of tissue cultures also leads to an increased activity of PAL [24], which is a link between primary and secondary metabolism, including many defense-related substances of the phenylpropanoid and flavonoid pathways. Furthermore, NIC treatment causes a pronounced increase in the levels of reduced and oxidized glutathione in tissue cultures of various plant species [9,10,24]. NIC-metabolites are also connected to plant stress- and defensive-metabolism; recently, it was shown that TRIG treatment of intact barley (*Hordeum vulgare*), by leaf spraying, caused an increased resistance against fungal infections in association with DNA-hypomethylation [25]. It is known that salt-stress can bring about an increased TRIG accumulation in the tissues of intact plants [26,27]. A role of TRIG as a osmoregulator and stress indicator has been suggested [27]. Here we show that treatment of plant tissue culture with the poly(ADP-ribose)polymerase (PADPRP) inhibitor 3-aminobenzamide (3-AB) counteracts stress-induced increases in PAL activity. These results, when taken together, indicate that PADPRP may influence the induction of defensive metabolism in plant tissues by stress.

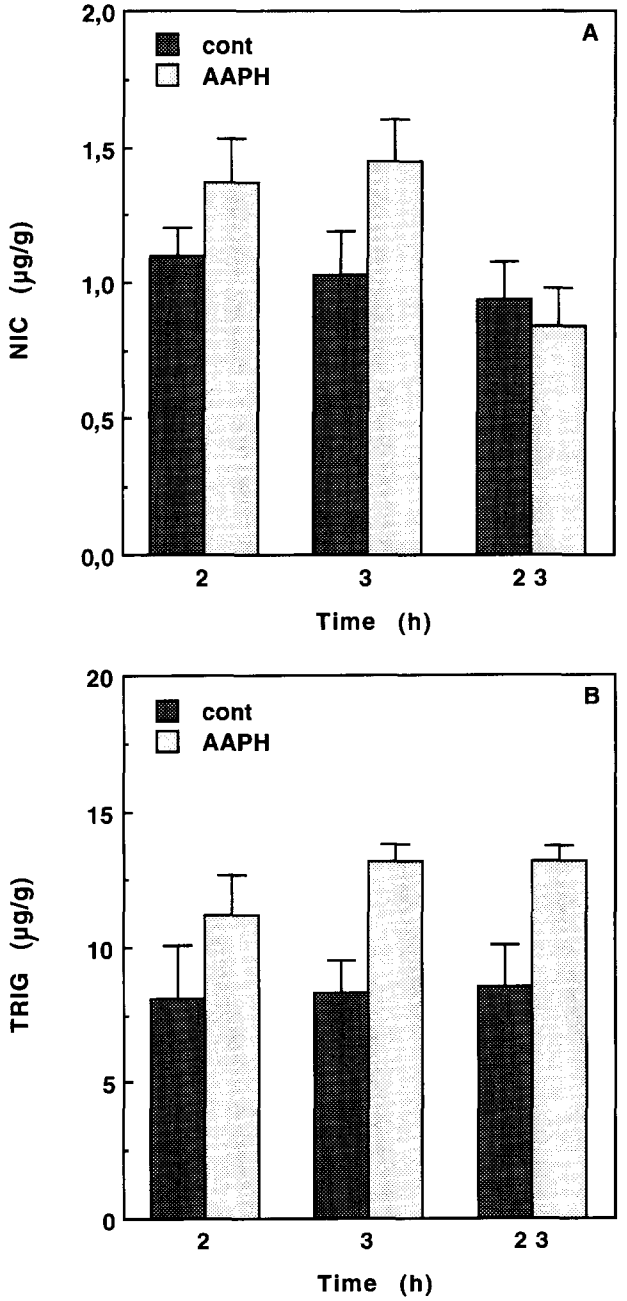

Fig. 1. Effect of 5 mM AAPH on the concentrations of (A) nicotinamide (NIC) and (B) trigonelline (TRIG) in *Catharanthus roseus* tissue culture CR32*. Addition of AAPH was made on day 17 in the growth cycle and cultures were harvested at time points after addition as indicated. Standard errors (n=2) are indicated.

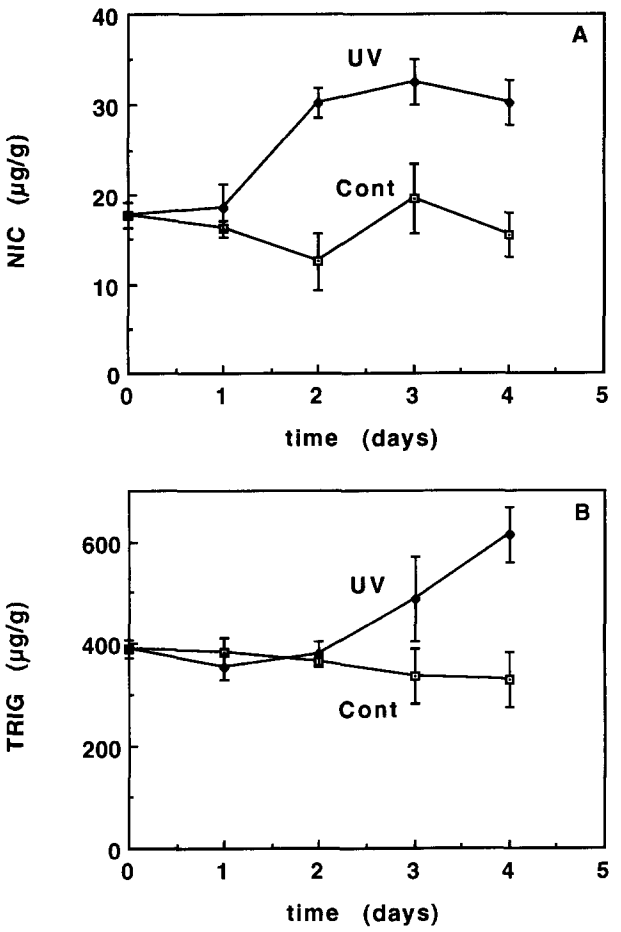

Fig. 2. Effect of supplementary UV-B radiation on the contents of (A) nicotinamide (NIC) and (B) trigonelline (TRIG) in *Pisum sativum* leaves. 17-day-old pea seedlings were either kept in the light regime in which they were grown (150 µE $m^{-2} \cdot s^{-1}$; control) or irradiated with supplementary UV-B radiation at the same PAR (UV) for the number of days indicated. Standard errors (n=2) are indicated.

2. Materials and methods

2.1. Cultures and culture conditions

The *Catharanthus roseus* culture CR32* and the anthocyanin-accumulating *C. roseus* culture CR19 PINK were grown in a modified B5-medium containing 3% sucrose, naphthalene acetic acid (NAA; 2 $mg \cdot l^{-1}$) and kinetin (0.05 $mg \cdot l^{-1}$) in a 12 h light/12 h dark regime, as described earlier [9].The cultures were harvested by vacuum filtration.

2.2. Growing of plants and UV-B treatment

P. sativum plants were grown and exposed to UV-B (biologically effective UV-B dose normalized to 300 nm: 60 kJ/m^2/day) radiation as described by Strid [18].

2.3. Chemicals

2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was provided from Wako Chemicals GmbH, Neuss, Germany. 3-Aminobenzamide (3-AB) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). AAPH, 3-AB and vanadylsulfate ($VOSO_4$) were dissolved in redistilled water and sterilized by filtration before addition to the cultures.

2.4. Determination of nicotinamide and trigonelline concentrations

Plant material (ca. 1 g) was accurately weighed, ground with sand and plunged into 3–4 ml of boiling water. The chilled material was extracted for 15–20 min three times with 3 ml of distilled water. The extracts were pooled and dried in a rotatory evaporator at room temperature. The dry matter was quantitatively taken up in 500 μl of water and 84 μl of this was spotted on SIL G/UV_{254} thin-layer plates, along with pure NIC and TRIG to facilitate component identification. Plates were developed in acetone/acetic acid/methanol/benzene (1:1:4:14 v/v) for localization of NIC and ethanol/1 M ammonium acetate (5:2 v/v) for localization of TRIG. After development, areas that corresponded to the two components were scraped from the plates and NIC and TRIG were extracted individually 4 times with water (300 μl). Quantitation of NIC and TRIG was accomplished by HPLC using a Waters μBondapac C_{18} reversed phase column and the conditions described by Miksic and Brown [28].

2.5. Phenylalanine ammonia-lyase determination

The activity of the enzyme phenylalanine ammonia-lyase was determined in crude extracts by the method described by Bolwell [29]. 1 g of tissue material (fresh weight) was extracted with 1 ml buffer solution. 1 Unit of activity (kat) was defined as the amount of enzyme which catalyzes the formation of 1 mole of cinnamic acid per second.

2.6. Glutathione determination

The contents of GSH_{tot} (the sum of GSH and GSSG) and GSSG were determined after extraction of 1 g fresh tissue material with 1 ml of 0.1 M HCl, using the Ellman's reagent according to the description by Brehe and Burch [30], as modified by Berglund et al. [9].

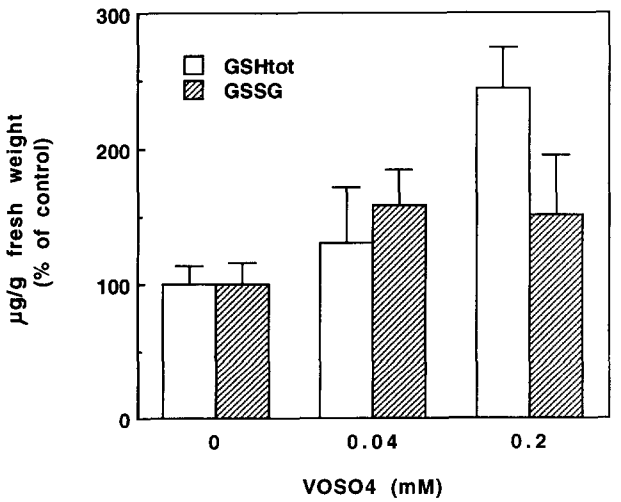

Fig. 3. Total glutathione (GSH_{tot}) and oxidized glutathione (GSSG) contents in *Catharanthus roseus* tissue culture CR19 PINK, treated with $VOSO_4$, added at day 12 in the growth cycle. Cultures were harvested 8 days after $VOSO_4$ addition. The control value for GSH_{tot} content was 12.8 μg·(g fresh weight)$^{-1}$ and for GSSG content 0.6 μg·(g fresh weight)$^{-1}$. Standard deviations (n=3) are indicated.

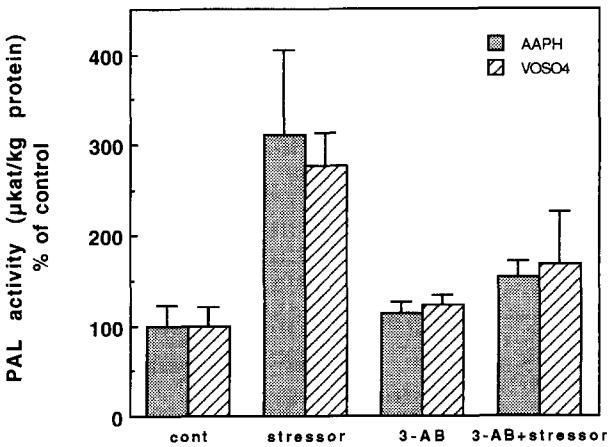

Fig. 4. Phenylalanine ammonia-lyase (PAL) activity in *Catharanthus roseus* tissue culture CR19 PINK after treatment with 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH; 2 mM) or vanadylsulfate ($VOSO_4$; 0.2 mM) in combination with 3-aminobenzamide (3-AB; 0.1 mM). In the AAPH-experiment, 3-AB and AAPH were added on day 11 and 12, respectively, and cultures were harvested on day 16 in the growth cycle. The control value for specific PAL activity was 11.0 μkat·(kg protein)$^{-1}$. In the $VOSO_4$-experiment, 3-AB and $VOSO_4$ were added on day 10 and 11, respectively, and cultures were harvested on day 14 in the growth cycle. The control value for specific PAL activity was 7.2 μkat·(kg protein)$^{-1}$. Standard deviations (n=3) are indicated.

3. Results

3.1. Nicotinamide and trigonelline

C. roseus tissue culture CR32* was treated with AAPH (5 mM) or $VOSO_4$ (0.4 mM) and analyzed for NIC and TRIG contents. The content of NIC, as well as TRIG were elevated to about 150% of the control value three hours after AAPH addition (Fig. 1A and B). After 23 h the content of NIC was decreased to the control level, while the TRIG content was maintained at a high level. $VOSO_4$ treatment of CR32* also caused a long-lasting elevation in TRIG content. Although the significance was low, the results indicated increased TRIG content after $VOSO_4$ treatment — about 25% increase after 3 h and about 50% increase after 23 h (not shown). However, no change in NIC content was seen (not shown).

NIC and TRIG contents in leaves of UV-B exposed *P. sativum* plants were increased as shown in Fig. 2A and B. The NIC content was elevated after 2 days of exposure and reached a maximum of approx. double the control value after 3 days, while the TRIG content increased first after 3 days and was even higher after 4 days of UV-B exposure.

3.2. Glutathione and PAL

$VOSO_4$ treatment of CR19 PINK resulted in dose-dependent increases in GSH_{tot} and GSSG levels (Fig. 3). The level of GSH_{tot} (total glutathione=the sum of reduced and oxidized forms) closely reflects the content of reduced glutathione (GSH), since the major part of glutathione in the cells is present in this form. The contents of GSH_{tot} increased to 250% and GSSG to 150% of the control levels by treatment with 0.2 mM $VOSO_4$ for 8 days. The elevating effect of AAPH on GSH_{tot} and GSSG contents in CR19 PINK and other plant tissue cultures has been shown earlier [20]. PAL activity in CR19 PINK was increased about three times by treatment

with 2 mM AAPH for 4 days or 0.2 mM $VOSO_4$ for 3 days (Fig. 4) and increased with time up to 8-fold, compared to the control value after 8 d of treatment with 1 mM AAPH [20].

3.3. 3-Aminobenzamide

C. roseus tissue culture CR19 PINK was treated with AAPH (2 mM) or $VOSO_4$ (0.2 mM), with or without pretreatment with 3-AB (0.1 mM) (Fig. 4). The PAL activity was increased to 300% of the control value by AAPH and 280% by $VOSO_4$. Pretreatment with 3-AB reduced the increases in PAL activity to 150% and 170% of the control, respectively. The GSSG content followed the same pattern as the PAL activity (not shown). 3-AB itself, at 0.1 mM, had no significant effect on PAL activity or GSSG content, but at 1 mM 3-AB both the PAL activity and the GSSG content decreased (Fig. 5). The relative decreases in PAL activity and GSSG content were about the same, irrespective of whether the cultures were harvested one or four days after 3-AB addition (Fig. 5). PAL activity was about 50% and GSSG content about 75–85% of the control values. The GSH_{tot} level was not influenced significantly by 0.1 mM or 1 mM 3-AB and 0.1 mM 3-AB did not affect the accumulation of anthocyanins in CR19PINK (not shown).

4. Discussion

4.1. NIC and TRIG levels

The NIC and TRIG contents in plant tissue cultures increased after AAPH treatment. However, after $VOSO_4$ treatment the level of NIC did not change, while there was an increase in TRIG level, similar to that observed after AAPH treatment. The reason for the absence of an increase in NIC after $VOSO_4$ treatment is not known, but may depend on the limited time points used in the experiment and a fast turnover of NIC. If the elevated NIC and TRIG levels are compared

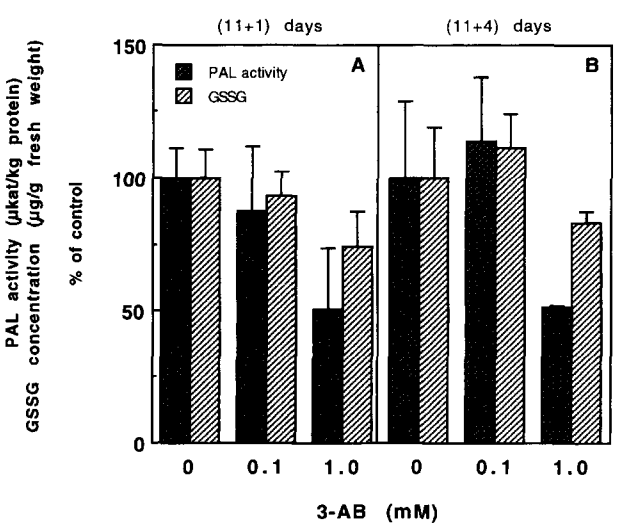

Fig. 5. Phenylalanine ammonia-lyase (PAL) activity and oxidized glutathione (GSSG) content in *Catharanthus roseus* tissue culture CR19 PINK, treated with 0.1 mM and 1.0 mM 3-aminobenzamide (3-AB). Additions were made at day 11 in the growth cycle and cultures were harvested (A) 1 and (B) 4 days after addition. Control values for specific PAL activity were 10.3 and 5.4 μkat·(kg protein)$^{-1}$, respectively, at the two harvest times. Standard deviations (n=3) are indicated.

with those after UV-B irradiation of intact leaves, a considerable difference in the onset of the increase is observed; there is a rapid increase of NIC and TRIG contents in the tissue culture, compared to the increase in leaves. One may speculate about the reasons for this. When considering the effect of AAPH on the glutathione levels in tissue culture, there is a rapid response; a decrease in the glutathione levels after about 3–9 h and an increase after about 9–12 h [31]. This is in line with a rapid increase in the levels of NIC and TRIG in plant tissue culture observed in the present study. Although transcription of defensive genes is rapidly induced in response to UV-B exposure [18,19], it may take some time before damage occurs, especially oxidative damage and excessive levels of thymine dimers, which may result in necrotic/apoptotic cell death. The exposure of plant tissue culture to water-soluble oxidative stressors in the growth medium is probably very efficient. Thus, one may expect that there is a more rapid occurence of damage in the AAPH- or $VOSO_4$-exposed tissue cultures than in the UV-B exposed leaves. The relatively strong UV-B radiation used in the present study caused considerable cell/tissue damage on prolonged exposure, which could promote PADPRP-mediated NIC release from NAD. This is in line with the suggestion by Willekens et al. [19] that certain parts of defensive metabolism occur in association with visible tissue damage. In line with the results by Green and Fluhr [32] we suggest that UV-B exposure in plants may cause increased levels of reactive oxygen species. Oxidative stress may cause DNA damage that is repaired by base excision repair [33], which in animal cells is considered to be associated with PADPRP activity [34]. However, stimulation of PADPRP activity did not seem to occur in response to UV-C exposure in animal cell systems [34].

The TRIG levels observed in the present study are in the range of those observed in other investigations [26]. However, for comparison there is little data on absolute NIC levels in plant tissue. The difference in NIC and TRIG levels between *C. roseus* tissue culture and *P. sativum* leaves may have various explanations, like differences between the types of tissue as well as water content or species diferences. The pronounced increase in TRIG content is in line with data from the literature, showing that NIC and nicotinic acid are rapidly metabolized to both TRIG and *N*-glucose nicotinic acid in intact plants, but to either TRIG or *N*-glucose nicotinic acid in undifferentiated plant tissue culture [35]. *N*-Glucose nicotinic acid was not investigated in the present study. Regarding the large stress-induced increase in TRIG content relative to the level of NIC, we are also aware of the possibility that a pool of *N*-glucose nicotinic acid, analogous to salicylic acid glucosides, could be a source of nicotinic acid and TRIG via stress-induced glucosidase and nicotinic acid *N*-methyltransferase activity. It has been proposed that green tissue/tissue-differentiation promotes NIC metabolization to TRIG [35]. The tissue culture CR32* used in the present investigation is partly green and differentiated and should be capable of TRIG biosynthesis, which was, indeed, shown in the present study. A role of chloroplast/plastid functions in the release and/or metabolism of NIC cannot be excluded.

There may be other cellular sources of NIC release as well. Cyclic ADP-ribose, which is involved in the regulation of cellular Ca^{2+} level by causing Ca^{2+} release from intracellular stores, is synthesized from NAD by NIC release [36]. Mono-ADP-ribosylations are NIC-releasing processes that may be

connected to stress, e.g. via ADP-ribosylation of GTP-binding proteins [37].

Information from the literature indicates other possible connections between UV-B exposure and NIC/TRIG metabolism. UV-B can increase the polyamine levels [8], which increase PADPRP activity [38], which can in turn result in elevated NIC and TRIG levels. In response to UV-B exposure the ABA levels are reported to increase [39]and ABA can stimulate TRIG accumulation [26].

The rise in TRIG in response to stress may be of paramount importance for the cellular control of DNA status. TRIG is known to cause arrest in the G2 phase of the cell cycle [40]. It is probably a good strategy to down-regulate mitotic activity in connection with stress which may be associated with DNA-damage. This arrest in G2 may give an opportunity to repair DNA damage and if damage is too severe, the cell can be killed in a programmed manner without excessive hazardous effects on surrounding cells. A role of PADPRP in the G2 checkpoint in mammalian cells has also been suggested [41].

4.2. $VOSO_4$

It has been suggested that vanadium compounds can cause an increased level of reactive oxygen species in plant cells [21]. Oxidation of vanadyl ions can generate hydroxyl radicals and other reactive oxygen species [23]. $VOSO_4$ is rapidly taken up by *C. roseus* tissue culture and was shown to increase the accumulation of indole alkaloid, partly derived from terpenoid, secondary metabolites [22]. The mechanisms behind these effects of vanadium compounds are not known in detail.

In tissue culture of the liverworth *Calypogeia granulata*, vanadate ion addition increased the accumulation of the terpenoid secondary metabolite 1,4-dimethylazulene and the activity of enzymes involved in the antioxidative defense (superoxide dismutase (SOD), ascorbate peroxidase, glutathione reductase (GR)) and in the level of reduced glutathione [21]. The increases in SOD and GR activities were rapid (within 2 h), which indicates a posttranscriptional activation. Furthermore, H_2O_2 addition increased the accumulation of 1,4-dimethylazulene in *C. granulata* [21] and it was suggested that reactive oxygen compounds contribute to the action of vanadate. The present study shows that $VOSO_4$ causes a strong increase in the levels of reduced and oxidized glutathione in plant tissue cultures, which indicates that reactive oxygen species are involved in the action of $VOSO_4$.

4.3. 3-Aminobenzamide

3-AB is frequently used as an inhibitor of the DNA strand break-induced enzyme poly(ADP-ribose)polymerase (PADPRP), especially in animal cell systems [42]. There are indications that PADPRP may influence secondary metabolism in plant tissues; when pigment-producing callus culture of red beet (*Beta vulgaris*) was treated with 3-AB (1 mM), there was a block in the pigment production [43]. Furthermore, it has been suggested that there is a connection between PADPRP and the expression of cytochrome P_{450} genes [44], because their expression was inhibited by PADPRP-inhibitor. Our present results, concerning 3-AB treatment (0.1 mM) of plant tissue cultures, and earlier results regarding effects of NIC indicate that PADPRP and one of its products, NIC, may constitute links in the signalling from stressor to the induction of defensive metabolism [9,10]. However, the site of action of 3-AB is not clear. Within the pathway from NAD to TRIG, there may be three potential 3-AB inhibited steps, i.e. those catalyzed by PADPRP, NAD glycohydrolase and nicotinamide amidohydrolase (nicotinamidase). There may be differences in the relative sensitivities of these steps to 3-AB. If the nicotinamidase is inhibited, there would be a rise in the NIC level and, possibly, the effects of NIC itself are observed (in contrast to those of the NIC metabolites nicotinic acid, *N*-glucose nicotinic acid and TRIG). Another inhibitor of PADPRP, 3-methoxybenzamide (3-MB), decreases the metabolism of NAD to TRIG, but there is an increase in the proportion of NIC [45]. This indicates that the metabolism of NIC to TRIG, probably by nicotinamidase, is a target of 3-MB, at least at a concentration of 0.1mM in intact non-stressed roots. The relative contributions of PADPRP and NAD glycohydrolase activity to NIC release from NAD in plants are not known. Even under non-stressed conditions there may be a considerable release of NIC by NAD glycohydrolase activity in association with a very high nicotinamidase activity [17,46]. The results in Fig. 5 show that the concentration of 3-AB (0.1 mM) used in the PAL induction study (Fig. 4) does not per se influence (decrease) the activity of PAL. This, in connection with decreased PAL induction in response to oxidative stressor when pretreated with 3-AB, is interpreted as an inhibitory effect of 3-AB on the induction of PAL activity in response to oxidative stressors. Thus, the counteracting effect of 3-AB on the induction of PAL by AAPH or $VOSO_4$ in the present study point to that PADPRP and/or nicotinamidase may be involved in stress-signalling by agents capable of causing oxidative stress. As mentioned earlier, we suggest that NIC functions as a signal in this process.

In conclusion, we suggest that PADPRP may be involved in the induction of plant defensive metabolism and that nicotinamide, released by either PADPRP or other NIC-releasing processes, may be involved in the regulation of plant defensive and secondary metabolism. We are convinced that future results will show the capacity of NIC and its metabolites to influence stress-connected/defensive metabolism in plant tissue.

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