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INHIBITION OF NITRATE AND NITRITE REDUCTASE INDUCTION IN WHEAT BY SANDOZ 9785

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Key Word Index—*Triticum vulgare* cv: HD 2189; Gramineae; wheat; nitrate reductase; nitrite reductase; SAN 9785; pyridazinone herbicide.

Abstract—The effect of a non-bleaching pyridazinone herbicide [4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone; SANDOZ 9785; BASF 13-338] on the induction of nitrate reductase (NR) and nitrite reductase (NIR) has been studied in leaf segments and seedlings of wheat. The induction of both NR and NIR was inhibited, albeit differentially, in detached green leaves as well as intact green seedlings grown in the presence of SAN 9785 and nitrate. The inhibition of NR and NIR induction due to SAN 9785 treatment occurred in the absence of photobleaching (detached leaves) or in the presence of marginal photobleaching (intact seedlings). Seedlings grown in the presence of SAN 9785 without nitrate showed a reduced capacity for induction of NR and NIR. The NADH-DCPIP reductase activity was not inhibited in SAN 9785-grown seedlings. The cause for inhibition of NR activity was neither inactivation of NR *in vivo* or *in vitro*, nor a reduction in the level of soluble protein. Both DCMU and SAN 9785 inhibited $^{14}CO_2$ fixation and induction of NR and NIR under similar conditions of treatment.

INTRODUCTION

In higher plants, the reduction of nitrate to nitrite occurs in the cytoplasm and is catalysed by the flavomolybdoprotein, NADH-nitrate reductase (NR; EC 1.6.6.1) and further reduction of nitrite to ammonia occurs in the chloroplast by ferredoxin-nitrite reductase (NIR; EC 1.7.7.1) [1–4]. The assimilatory nitrate reductase holoenzyme NADH-NR of eukaryotic organisms is composed of FAD-containing NADH-cytochrome *c* dehydrogenase (diaphorase) moiety and Mo-containing terminal nitrate reductase moiety, both of which participate in the two electron transfer from NADH to nitrate [3]. Recent evidence indicates that the bulk of the nitrate reductase is located in the cytoplasm and some is associated with the plastids [5]. Its apoprotein is encoded in a nuclear gene and the translation of its *m*RNA occurs on cytoplasmic ribosomes [6]. Recently, considerable nitrite reductase activity has been reported from ribosome-deficient plastids and extracts of white seedlings (plastome mutants) of barley and, based on this observation, it has been suggested that this enzyme is also synthesized in the cytoplasm [7]. The low activities of NR and NIR observed in pigment-deficient leaves of the chloroplast ribosome-deficient mutants of barley and maize [7], albino mutants of barley [8, 9], and norflurazon bleached leaves of barley [10] suggest that chloroplast structural integrity is a prerequisite for optimal activities of NR and NIR.

Recently, it has been reported that photooxidative damage to plastids caused by exposing etiolated norflurazon (SAN 9789)-treated mustard seedlings to white light resulted in (i) a loss of activities of NIR and some plastidic enzymes in 6–12 hr; (ii) loss of NIR inducibility by nitrate after 2 hr and (iii) almost 50% inhibition of NR inducibility after 0.5 hr of photooxidative treatment [11]. Photooxidative damage appeared to affect and eliminate some 'plastidic signal' that is responsible for the formation of NR and NIR. SAN 9789 is a bleaching herbicide and when seedlings are grown in the presence of the herbicide in light, photobleaching occurs [12]. The effects of SAN 9789 become more complex and drastic when etiolated seedlings are exposed to the herbicide in light because the development of the etioplast into chloroplast also is severely inhibited [11]. It is difficult to ascertain whether the inhibition of NIR and NR induction by SAN 9789 [11] is due to photooxidative damage or prevention of etioplast development into chloroplast. We have therefore employed for the present investigation a non-bleaching herbicide of the same family, SAN 9785 [4-chloro-5-(dimethylamino)-2-phenyl-3(2H)-pyridazinone] and mature green leaves. Our present results show that induction of NR and NIR can also be inhibited by treatment with a non-bleaching pyridazinone herbicide.

RESULTS

When leaf segments from seven-day-old green seedlings were exposed to nitrate and SAN 9785 in light, the induction of NR and NIR was inhibited, albeit differentially, at all concentrations of the herbicide employed. At 125 μM SAN 9785, NIR activity was inhibited 42% as compared to 28% inhibition of NR activity. However, with increasing herbicide concentration, the inhibition of NR activity became more than that of NIR (Fig. 1). At 500 μM SAN 9785, the inhibition of NR activity was 72% against 55% for NIR activity. The I_{50} values for NR and

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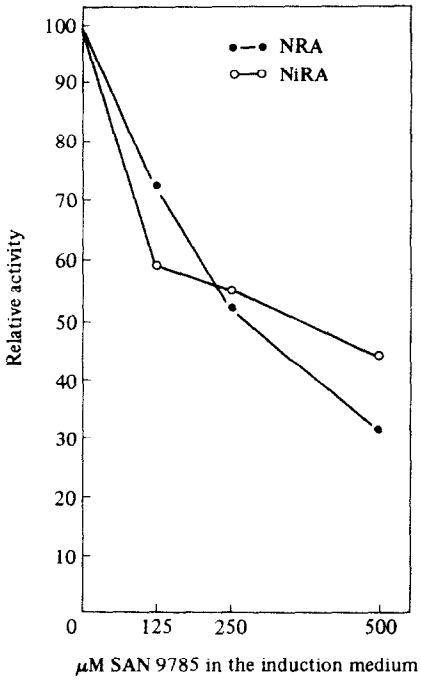

Fig. 1. Effect of SAN 9785 on the induction of NR and NIR in wheat leaf segments. Wheat seedlings were raised for 7 days in light in distilled water. Leaf segments, 4 cm long, were floated on 60 mM potassium nitrate (control) and 60 mM potassium nitrate plus varying concentrations of SAN 9785 in light for 18 hr. Activities of NR and NIR were estimated by the *in vitro* method. The control (100%) values for NR and NIR activities were 3.46 μmol NO_2^- formed/hr/g fr. wt and 166 μmol NO_2^- reduced/hr/g fr. wt, respectively.

NIR were 250 and 350 μM, respectively. There was no change in chlorophyll content in the leaf segments exposed to the herbicide (data not shown).

To check the effect of SAN 9785 on the induction of NR and NIR in intact seedlings grown in the presence of the herbicide, seven-day-old seedlings were raised from seed in light in the presence of nitrate and the herbicide. Seedlings grown in 125 μM SAN 9785 lost only 12% of their chlorophyll but 60% of their *in vitro* NR and 20% of NIR activities. Whereas the reduction in chlorophyll level was 15 and 20% in seedlings grown in 250 and 500 μM SAN 9785, respectively, the inhibition of NR induction was 75% and 90%, respectively, and that of NIR was 45% and 75%, respectively (Table 1). Interestingly, the I_{50} values decreased to 100 and 300 μM for NR and NIR activities, respectively, in herbicide-grown intact seedlings (Fig. 2).

Regardless of whether nitrate was added to seedlings along with the herbicide during induction or subsequent to growing the seedlings in the herbicide, induction of NR as well as NIR was suppressed. When seedlings grown in the presence of the herbicide were subsequently exposed to nitrate, the induction of NR and NIR was suppressed by 63 and 29%, respectively (Table 2). Seedlings grown in 0.5 mM SAN 9785 showed 63 and 29% inhibition of NR and NIR activities, respectively, without any inhibition of diaphorase activity (Table 2).

Table 1. Effect of SAN 9785 on chlorophyll level in seven-day-old wheat seedlings

SAN 9785 (μM)	Total chlorophyll (mg/g fr. wt)	Total chlorophyll as % of control
0	1.95	100
125	1.71	88
250	1.65	85
500	1.58	81

The details of herbicide treatment are as given in the Experimental.

Table 2. Effect of pretreatment with SAN 9785 on the *in vitro* NR, NIR and diaphorase activities in wheat seedlings

Treatment	NR activity	NIR activity	Diaphorase activity, ΔA_{610}
Control	2.40	176	0.415
SAN 9785	0.887	125	0.410

Wheat seedlings, seven-day-old, were raised in distilled water or 0.5 mM SAN 9785. Seedlings were provided with 15 mM potassium nitrate 36 hr before harvest for assay.

NR activity; μmol nitrite produced/hr/g fr. wt

NIR activity: μmol/nitrite reduced/hr/g fr. wt

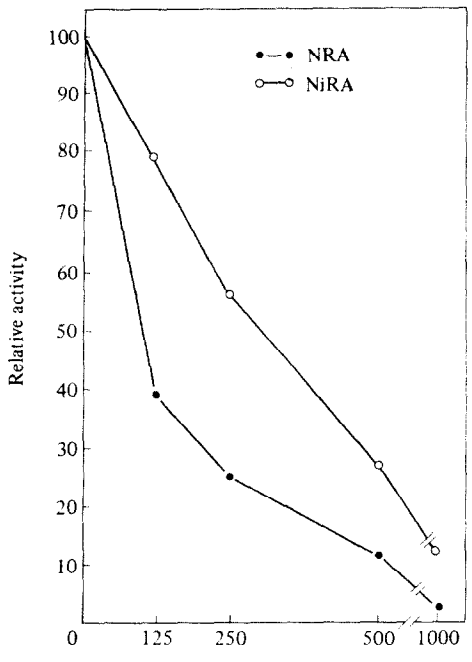

Fig. 2. Effect of SAN 9785 on the induction of NR and NIR in intact wheat seedlings. Wheat seedlings were raised in 15 mM potassium nitrate (control) and 15 mM potassium nitrate plus different concentrations of SAN 9785 for 7 days in light. The 100% activities of NR and NIR (control) were 3.73 μmol NO_2^- formed/hr/g fr. wt and 136 μmol NO_2^- reduced/hr/g fr. wt, respectively.

Table 3. Effect of DCMU and SAN 9785 on $^{14}CO_2$ fixation and induction of NR and NIR

Treatment*	CPM/mg fr. wt	% Rate
Control	4610	100
200 μM DCMU	640	14
1 mM SAN 9785	1010	22
Treatment†	*In vitro* NR activity	NIR activity
Control	100	100
100 μM DCMU	0	20

*The details are as given in the Experimental.

† Leaf segments, 4 cm long, from seedlings grown in water were floated in 60 mM potassium nitrate (control) and 60 mM potassium nitrate + 0.1 mM DCMU for 24 hr in light. The leaf segments were washed before enzyme extraction. The control (100%) values for activities of NR and NIR were 4.15 μmol NO_2^- formed/hr/g fr. wt and 116 μmol NO_2^- reduced/hr/g fr. wt, respectively.

In order to check whether the herbicide inactivated the NR *in vivo*, varying amounts of the herbicide were added to the *in vivo* NR assay medium containing leaf segments from seven-day-old green seedlings grown in 15 mM potassium nitrate. No inactivation of the enzyme was observed in leaf segments even after 1 hr exposure to 1 mM herbicide. Similarly, when the isolated enzyme (crude leaf extract) from the leaves of these plants was incubated on ice for 1 hr with different amounts of the herbicide (100–500 μM), no inactivation of the enzyme was noticed (data not shown). Further, the possibility that the lowered enzyme activity may have resulted from suppression of protein synthesis by the herbicide was examined. The results have shown that the soluble protein levels in the leaves of plants grown in varying concentrations (125–500 μM) of the herbicide did not vary markedly (data not shown).

Recent observations indicate that SAN 9785 induces changes in structure and function of wheat chloroplasts and that it is a DCMU-type inhibitor [13–15]. It has been shown that DCMU, an inhibitor of non-cyclic electron transport, inhibited the induction of NR and NIR in green leaves of rice [16]. In the present investigation on green leaf segments of wheat, DCMU (200 μM) caused 86% inhibition of $^{14}CO_2$ fixation, while SAN 9785 (1 mM) caused 78% inhibition (Table 3). Under similar experimental conditions, 100 μM DCMU inhibited the induction of NR completely, while inhibiting 80% of NIR induction (Table 3).

DISCUSSION

The data presented in this paper show that SAN 9785 effectively inhibits the induction of NR and NIR in detached green leaves (Fig. 1) and intact seedlings (Fig. 2) of wheat. Reports indicating the dependence of NR and NIR induction on chloroplast integrity are usually from investigations employing either ribosome-deficient plastids [7], albino mutants [8, 9], or norflurazon-bleached plants [10, 11], in which the chloroplast development was blocked or rudimentary. By contrast, our present results with a non-bleaching pyridazinone herbicide, SAN 9785, and green wheat leaf segments with normally developed chloroplasts show that neither photooxidative bleaching nor drastically impaired chloroplast development is prerequisite to inhibition of NR and NIR induction.

Though the primary effect of SAN 9785 has been shown to be upon desaturation of linolenic acid in photosynthetic tissues [17–20], it has also been shown to inhibit oxygen evolution [21, 22] and cause subtle changes in thylakoid membrane organization [13, 15, 21, 23, 24]. The inhibition of $^{14}CO_2$ fixation and induction of NR and NIR by both DCMU and SAN 9785 observed in the present investigation is in agreement with the earlier observations that SAN 9785 is a DCMU-type inhibitor [14, 24]. However, the inhibition of NR and NIR induction by SAN 9785 is noteworthy in view of the reported lack of inhibition of the content and functioning of RuBP case [25] and activities of other Calvin cycle enzymes [26] in wheat seedlings. Further, the inhibition of NR and NIR induction is not parallel in the sense that NR induction is inhibited more than that of NIR under similar conditions of treatment (Figs 1, 2). From this observation, it may be speculated that SAN 9785 has different sites of action, because NR is considered to be a cytoplasmic enzyme whereas NIR is a chloroplastic enzyme. That the primary effect of SAN 9785 need not be restricted to chloroplasts has become evident from the recent report that SAN 9785 decreases the linolenate content of phosphatidylchloline involved in the synthesis of storage lipids of cotyledons [27].

It has been shown that the level of NR is regulated by *de novo* synthesis and protein degradation [28]. The absence of NR inactivation *in vivo* or *in vitro* and of marked alterations in the soluble protein level following SAN 9785 treatment rule out the possibility of herbicide inactivation of the enzyme *per se* or a general decline in cytoplasmic protein synthesis as being the causative factors for the decrease in NR induction. NR induction has been shown to depend on respiration [29–31]. The suppression of NR and NIR induction in wheat leaves treated with DCMU (Table 3) and SAN 9785 (Fig. 1) may be ascribed to the lowered CO_2 fixation [Table 3; 24, 26] as a result of inhibition of non-cyclic electron transport and consequent decrease in the availability of respirable carbohydrates. Individually or in concert, inhibition of respiration [26] could be another causative factor.

Suppression of NR and NIR induction in seedlings fertilized with nitrate after being grown in the herbicide for seven days (Table 2) may be attributed to the cellular alterations that the herbicide may have brought about leading to decreased capacity for NR and NIR synthesis. Recently, the involvement of a 'plastidic signal' in the regulation of nuclear gene expression including the synthesis of NR and NIR has been suggested [11]. Also, the 'plastid factor' has been suggested to act on NR-apoprotein accumulation [7]. It remains to be explored whether SAN 9785 *per se* affects nuclear gene expression and eliminates or damages the proposed 'plastid factor' involved in the synthesis of NR and NIR. Our results show that at least the FAD-containing diaphorase moiety is not affected by SAN 9785 treatment (Table 2).

EXPERIMENTAL

Plant material. Seeds of *Triticum vulgare* cv HD 2189 were surface sterilized for 1 min with 0.1% $HgCl_2$ and were allowed to

germinate at room temp. (30±2°) on 3 layers of coarse filter paper in a glass Petri plate. For growing seedlings in SAN 9875, various concns of the herbicide (125, 250 and 500 μM) were prepared in 0.5% acetone and 20 ml of each soln was added initially. Subsequently, the seedlings were provided with only tap H_2O periodically. Illumination was provided by a bank of 4 cool daylight fluorescent lamps (Philips, India). Seedlings were grown for 7 days under a 14 hr day/10 hr night regime.

Enzyme extraction and assay. Leaves of uniform size were cut into small segments and homogenized with a chilled pestle and mortar in 6 vol. of ice-cold extraction medium consisting of 25 mM KH_2PO_4–KOH, pH 7.5; 5 mM dithiothreitol and 5 mM EDTA. The brei was centrifuged at 27000 *g* for 15 min at 4° (Sorvall, Model RC-5B, U.S.A.). The supernatant was stored on ice prior to assaying the enzymes. Enzymes were assayed under conditions such that the rate of reaction was proportional to time and amount of enzyme at 30° and pH 7.5. Assays in triplicate were made on each sample.

NR assay method (in vitro). The reaction mixture contained, in a final vol. of 2 ml, 100 μmol KH_2PO_4–KOH, pH 7.5, 20 μmol KNO_3, 0.8 μmol NADH, 0.2 ml enzyme and H_2O. A minus-NADH reaction mixture was used as control. Unless otherwise stated, the reaction time was 15 min at 30°. The post-assay treatment was carried out according to [32]. The reaction was stopped by adding 0.2 ml of 1 M zinc acetate followed by 0.2 ml of 0.15 mM phenazine methosulphate to oxidize the residual NADH. After 20 min, the reaction mixture was centrifuged, and to the supernatant were added in quick succession 1 ml of 1% (w/v) sulphanilamide reagent prepared in 3 M HCl and 1 ml of 0.02% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride reagent. The colour was allowed to develop for 15 min prior to reading at 540 nm in a Gilford 250 Spectrophotometer. The amount of nitrite formed was calculated based on E^{1mM}_{1cm} nitrite complex (540 nm) = 55. Enzyme activity was expressed as μmol of nitrite produced equivalent fr. wt/hr.

NADH-DCPIP reductase (diaphorase). The assay method is essentially similar to that described in [33].

MV-nitrite reductase. The enzyme activity was determined by the method of [16].

NR assay method (in vivo). The assay was performed as described in [34] except that Neutronyx 600 was substituted with 1% (v/v) *n*-propanol and 0.1% (v/v) Triton X-100.

$^{14}CO_2$ fixation by control and herbicide-treated leaves. Leaves of 7-day-old wheat seedlings grown in H_2O were incubated for 5 min under illumination in 50 mM KH_2PO_4–KOH buffer, pH 7.5 containing 5 mM $MgCl_2$, 35 mM NaCl and 10 mM $NaHCO_3$ ± additives (DCMU or SAN 9785). $NaH^{14}CO_3$ was injected into the reaction mixture and incubated for 30 min in light. The reaction was arrested by cold HOAc (final concn 10%). The leaf segments were washed and ground in incubation medium and the vol. was made up to 3 ml. Aliquots of 100 μl of the homogenate were loaded onto 4 Whatman No. 1 filter paper discs and dried at room temp. under incandescent lights. The radioactive carbon fixed was measured by scintillation spectroscopy (Packard, Tricarb C2425).

Chlorophylls were extracted in 80% Me_2CO and the concn was calculated according to ref. [35]. Protein in crude enzyme extracts was estimated according to ref. [36].

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