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EFFECT OF *N,N*-DIALLYL-2,2-DICHLOROACETAMIDE (R-25788) ON EFFLUX AND SYNTHESIS OF GLUTATHIONE IN TOBACCO SUSPENSION CULTURES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; glutathione; glutathione synthesis; herbicide antidotes; tissue culture.

Abstract—The low level of glutathione synthesis observed in tobacco suspension cultures grown under heterotrophic conditions was stimulated by the addition of the antidote R-25788 to the culture medium. Treatment of the cells with 10^{-5} M R-25788 enhanced the amount of glutathione released during the whole culture period of 12 days by a factor of 1.7–1.8, without affecting the rate of efflux or the intracellular glutathione content. Stimulation of the high level of glutathione synthesis observed in photoheterophically grown suspensions was not achieved by the antidote. Whereas addition of R-25788 to the assay mixture did not increase glutathione synthesis *in vitro*, the specific activity of the enzymes of glutathione synthesis was enhanced to a significant extent in tobacco cells, cultured in medium containing R-25788. Therefore, R-25788 seems to stimulate glutathione synthesis by an enhancement of the amount of enzymes involved in this process, rather than by a direct activation of pre-existing enzymes.

INTRODUCTION

Protection of crop plants from herbicide injury is not only achieved by developing herbicides with selective phytotoxicity, but also by the use of chemical antidotes [1, 2]. The term 'antidote' refers to substances which prevent herbicide injury to crop plants without reducing the activity of the herbicide to other plant species. *N,N*-Diallyl-2,2-dichloroacetamide (R-25788) is such a physiologically selective antidote that protects corn plants from the action of EPTC (*S*-ethyl *N,N*-dipropylthiocarbamate) [3, 4] and many other thiocarbamate herbicides [5]. As it has been clearly shown that R-25788 does not protect corn plants by preventing EPTC uptake or translocation [6, 7], this antidote seems to affect the metabolism of EPTC.

In earlier studies, hydrolysis to ethylmercaptan, carbon dioxide and dipropylamine has been proposed for the degradation of EPTC in plant cells [8, 9]. However, recent investigations with corn roots indicate that thiocarbamate herbicides like EPTC can be converted to their corresponding sulfoxides [7, 10, 11]. These sulfoxides seem to be the primary phytotoxic compounds, due to their potential to carbamoylate important thiols like glutathione and coenzyme A [7]. EPTC-sulfoxide may be detoxified by either spontaneous [12] or enzymological [7] conjugation with glutathione to yield *S*-(*N,N*-dipropylcarbamyl)-glutathione [11].

Treatment of corn roots with R-25788 results in an increased glutathione content [7, 13] and an enhanced glutathione-*S*-transferase activity of the cells [7]. Therefore in the presence of R-25788 detoxification of EPTC by conjugation with glutathione may occur, while sufficient glutathione still remains to participate in other metabolic processes. Carringer *et al.* [13] recently showed that the activity of the enzymes of glutathione synthesis is stimulated, *in vitro*, by addition of R-25788 to the assay mixture; but no effect on the activity of these enzymes was observed in homogenates of corn roots that were pretreated with the antidote. Although an R-25788 mediated increase in the glutathione content has been demonstrated in several other species [14], there is still no direct evidence for an effect of R-25788 on the enzymes of glutathione synthesis *in vivo*. The present experiments were performed under the controlled conditions of tissue culture to obtain information concerning such an effect of R-25788. Suspension cultures of *Nicotiana tabacum* var. Samsun were chosen for this study because of their potential to produce high amounts of glutathione under known physiological conditions [15–17].

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RESULTS AND DISCUSSION

Treatment of heterotrophically grown tobacco cells with 10^{-7}–10^{-5} M R-25788 stimulates the efflux of glutathione into the culture medium. As shown in Fig. 1(A) the medium in which cell suspensions were grown with 10^{-5} M R-25788 for 10 days, contained *ca* 180% of the amount of glutathione found in controls without antidote. The investigation of the pattern of the release of glutathione into the medium during a culture period of 12 days showed that, although the rate of efflux is not changed by treatment of the tobacco suspensions with R-25788 under heterotrophic conditions, the amount of glutathione released by antidote-treated tobacco cells is enhanced 1.7–1.8-fold at each time of culture (Fig. 2A). As the growth of the cultures is not influenced to any significant extent by exposure to R-25788 (Fig. 1A; Fig.

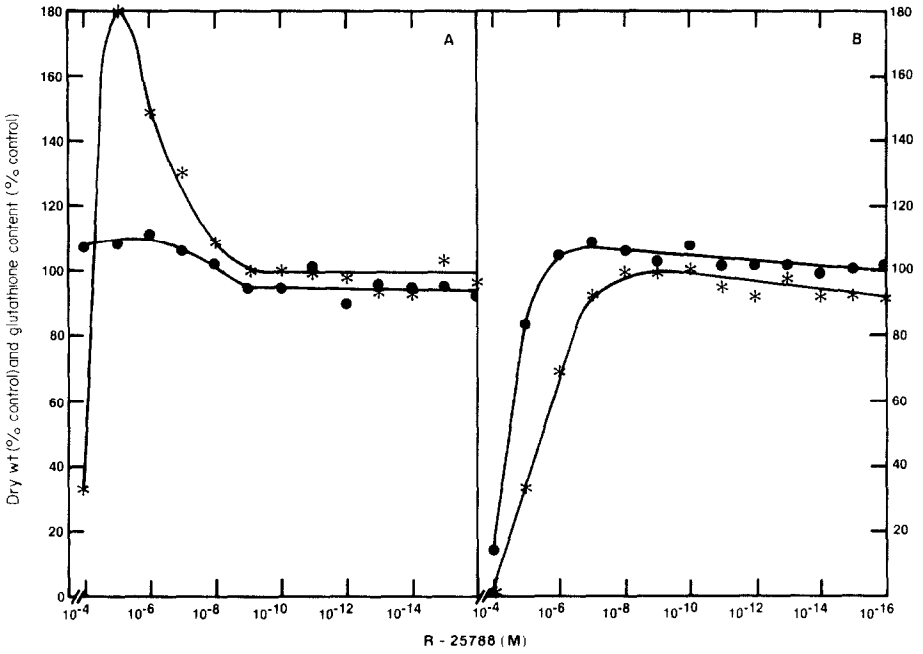

Fig. 1. Influence of R-25788 on growth and efflux of glutathione in heterotrophically and photoheterotrophically grown tobacco suspension cultures. 1.8 mg dry wt (±10%) of dark-grown (A) and light-grown (B) tobacco cells were inoculated into 30 ml portions of culture media containing the R-25788 concentrations indicated. After 10 days of culture in the dark (A) or in the light (B) dry wt yield (●—·—●) and glutathione content of the medium (*— *) were determined and compared with controls minus antidote.

2A), these data suggest an influence of the antidote on the capacity for glutathione synthesis.

In photoheterophically grown tobacco suspensions that release 12–14-fold higher amounts of glutathione into the culture medium than heterotrophically grown cells, a R-25788 mediated stimulation of the efflux of glutathione was not observed (Fig. 1B; Fig. 2B). In these cultures, antidote concentrations higher than 10^{-8} M reduce the release of glutathione, whereas treatment with smaller concentrations does not influence the efflux of this peptide (Fig. 1B). In addition, the pattern of growth and efflux of glutathione are not affected by R-25788 in photoheterotrophically grown tobacco cultures (Fig. 1B).

The enhanced accumulation of glutathione in the medium of tobacco suspensions, grown with R-25788 under heterotrophic conditions, is not only due to a stimulation of the release of glutathione, but also to increased synthesis of this compound. Irrespective of the glutathione content of the culture medium, the amount of glutathione remaining inside heterotrophically grown tobacco cells is not influenced by treatment of the suspensions with the antidote R-25788 (Table 1). Also in photoheterotrophically grown tobacco cells, the glutathione content is not affected by this compound (Table 1). These observations show that (1) the enhanced glutathione content in the medium of R-25788 treated,

Table 1. Comparison of the effect of R-25788 on the intracellular and the extracellular glutathione content of tobacco suspension cultures

Age of culture (days)	Glutathione in the medium of cultures $\left(\frac{+\text{R-25788}}{-\text{R-25788}}\right)^*$		Glutathione in the cells of cultures $\left(\frac{-\text{R-25788}}{+\text{R-25788}}\right)^*$	
	Photoheterotrophic	Heterotrophic	Photoheterotrophic	Heterotrophic
4	0.93	1.75	—	—
6	1.13	1.80	1.00	1.04
8	0.98	1.84	1.04	1.07
10	1.15	1.79	0.94	1.06
12	0.96	1.71	0.86	0.91

Tobacco cells were inoculated and cultured as described in Fig. 2. After the culture periods indicated, the glutathione content of the medium and the cells was determined.

*A ratio of 1.00 indicates no effect of the antidote R-25788.

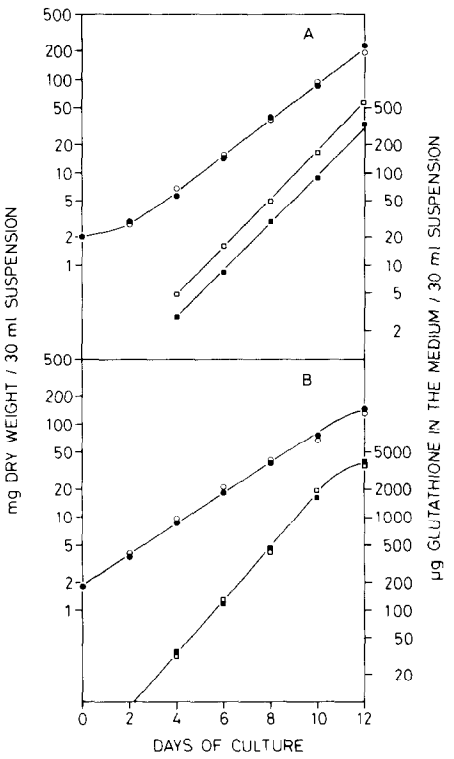

Fig. 2. Pattern of growth and efflux of glutathione in heterotrophically and photoheterotrophically grown tobacco suspensions treated with R-25788. 1.8 mg dry wt (±10%) of heterotrophically grown tobacco cells were inoculated into 30 ml portions of culture medium containing 10^{-5} M R-25788 (A), 1.7–2.0 mg dry wt of photoheterotrophically grown tobacco cells into medium containing 10^{-9} M R-25788 (B). Controls were grown in R-25788 free medium. After the culture periods indicated, dry wt yield and glutathione content of the medium was measured. ●——●, Dry wt of untreated cells; ○——○, dry wt of R-25788 treated cells; ■——■, glutathione content of the medium of untreated suspensions; □——□, glutathione content of the medium of R-25788 treated suspensions.

heterotrophically grown tobacco suspensions is not caused by leaky cells, and (2) the lack of an R-25788 mediated enhancement of the glutathione content in the medium of photoheterotrophically grown cells is not accompanied by an accumulation of glutathione inside the cells, due to a limiting capacity of the translocation system of this peptide. Such an accumulation has been observed in green tobacco cells, grown on solid culture medium (H. Rennenberg, unpublished results). Therefore, further stimulation of the high level of glutathione synthesis taking place in green tobacco cells in suspension culture is not achieved by R-25788, whereas the low level of glutathione synthesis in tobacco cells, grown under heterotrophic conditions, can be stimulated by this compound.

A R-25788 mediated stimulation of glutathione synthesis in crude corn root homogenates has been reported to be due to a direct activation of the pre-existing enzymes involved in this pathway [13]. In the present investigation no synthesis of glutathione was obtained in the test tube system [22] using crude tobacco cell homogenates. In tobacco cell preparations that were purified on Biogel P-2, glutathione synthesis was not activated by addition of 10^{-8}–10^{-4} M R-25788 to the assay mixture. However, the specific activity of the enzymes of glutathione synthesis is enhanced to a significant extent in tobacco cells, grown heterotrophically in R-25788 containing culture medium (Table 2). These data indicate that the stimulation of glutathione synthesis by R-25788 in heterotrophically grown tobacco cells is not caused by a direct activation of pre-existing enzymes, but by an enhancement in the amount of enzymes involved in this process. Further investigations will show whether this enhancement is caused by *de novo* synthesis or an indirect activation of pre-existing enzymes.

Table 2. Effect of R-25788 on the specific activity of the enzymes of glutathione synthesis in heterotrophically grown suspension cultures

Age of culture (days)	Sp. activity (µmol glutathione/min/g fr. wt)	
	− R-25788	+ R-25788
4	15.8	31.7
8	17.2	32.0
10	12.4	24.6

Tobacco cells were inoculated and grown heterophically as described in Fig. 2(A). After the culture periods indicated, cells were homogenized, the extracts purified on Biogel P-2, and the activity of the enzymes of glutathione synthesis determined.

EXPERIMENTAL

The tobacco suspension culture used in the present investigation was obtained from a callus culture of *N. tabacum* var. 'Samsun' isolated by Bergmann [18]. Cells were subcultured into a modified, liquid MS medium [19], and grown at 25° and 60–70% air humidity either under continuous illumination (3000 lx) or on an orbital shaker (100 rpm) in the dark. Experiments were performed with tobacco cells in the exponential phase of growth; R-25788 was added to the media as a filter-sterilized solution at pH 5.6–5.8 under sterile conditions.

Glutathione in conditioned media and in tobacco cell homogenates was quantified as the σ-phthalaldehyde derivative [20] after purification of the samples by ion-exchange and TLC [16]. As a growth parameter the dry wt content of the cells was measured by the method reported by Bergmann *et al.* [21]. The activity of the enzymes of glutathione synthesis was determined as the amount of ^{14}C-labelled glutathione formed from glutamic acid, cysteine, and ^{14}C-labelled glycine (B. Schaer, unpublished results) in tobacco cell homogenates that had been purified on Biogel P-2.

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