Phytochemistry, Vol. 26, No. 10, pp. 2735–2739, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

THE PRODUCTION OF SECONDARY METABOLITES BY *DIGITALIS LANATA* DURING CO_2 ENRICHMENT AND WATER STRESS

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(*Revised received* 7 *April* 1987)

Key Word Index—*Digitalis lanata*; Scrophulariaceae; woolly foxglove; CO_2 enrichment; leaf water potential; cardenolides; digoxin.

Abstract—The influence of atmospheric CO_2 enrichment and water stress on the production of biomass and cardioactive substances by the woolly foxglove *Digitalis lanata* was investigated. Carbon dioxide enrichment (1000 ppm) had a 'fertilizing' effect in that both biomass and cardenolide content increased to about 160% of the control. The yield of the pharmacologically relevant major product, digoxin, significantly increased following enrichment, whereas two other compounds decreased. Water stress, in the physiological range, reduced fresh weight more than either cardenolide content or dry weight. The amount of digitoxigenin was considerably reduced, whereas the other cardenolides, including digoxin, were less affected. CO_2-enriched plants, which were also subjected to drought, exhibited mixed responses. We conclude from these investigations that not only primary, but also secondary metabolism is influenced by variations of the environment. Possible ecological consequences of changes in secondary metabolism due to atmospheric CO_2 enrichment and water stress are discussed.

INTRODUCTION

Secondary plant metabolites are often essential components of a plants protective reactions against herbivores or invasion by microorganisms. If variations of the abiotic environment could change the production of such metabolites, ecological and in some cases economic consequences, would follow.

In order to investigate the responses of secondary metabolism to water stress and permanent CO_2 enrichment, we used *Digitalis lanata* EHRH. This species always produces a variety of protective substances, the cardenolides, almost all of which have structurally been identified [1]. The major substances are indispensible cardioactive medicines in the treatment of cardiac insufficiency. Because of the production of the potent 'C-series' glycosides, *D. lanata* is pharmaceutically more important than *D. purpurea*.

Up to now, neither synthetic chemicals nor cell cultures have been able to compete economically with agricultural cardenolide production though the biotransformation of digitoxin to digoxin [2] seems to offer the potential for commercial exploitation. By selection it has been possible to produce chemocultivars of *Digitalis* which have a high level of the desired cardenolide [3, 4]. Parental selection has led to a high digoxin yield from the cultivar of *D. lanata* used in these investigations, but glycosides hydroxylated in position 16 (B, D, and E series) are almost completely absent. A suppression of the A series cardenolides, which are biosynthetically close related to the digoxin 'C' series (Fig. 1), is less expressed. The relatively large amounts of the aglycone digitoxigenin found may be attributed to phytotron cultivation, since in field grown plants the proportion of digitoxigenin is smaller (data not shown).

As in the case of the production of pharmaceutical preparations, the number of glycosides was reduced during these experimental procedures by deglucosylation and deacylation. This led to an almost complete transformation of a primary glycoside to the corresponding secondary glycoside. The quantification of the maximum yield of deglucosylated compounds and especially of the pharmaceutically relevant secondary glycosides was thus achieved.

The influence of water stress on cardenolide synthesis in *D. lanata* has been studied previously [5]. Much of the data, however, was from a non-physiological range of water stress from which the plants were unable to recover. We adapted an alternative approach in that we were interested in ecological features.

In addition to the stress investigation, we studied the response of secondary metabolism to CO_2 enrichment. The concentration of CO_2 in the natural atmosphere has risen from about 290 ppm in the pre-industrial period [6] to 350 ppm up to now and, from our present knowledge, could rise even higher in the future [7]. Although a growth-enhancing effect of additional CO_2 in C_3 plants is well established, almost nothing is known of its effect on the synthesis of secondary compounds. We have given special attention to biomass production and the synthesis of cardenolides under CO_2 enrichment, a single period of drought stress, and a combination of these two factors.

RESULTS AND DISCUSSION

Responses during CO_2 enrichment

Atmospheric CO_2 enrichment to 1000 ppm (continuously maintained throughout plant growth) enhanced

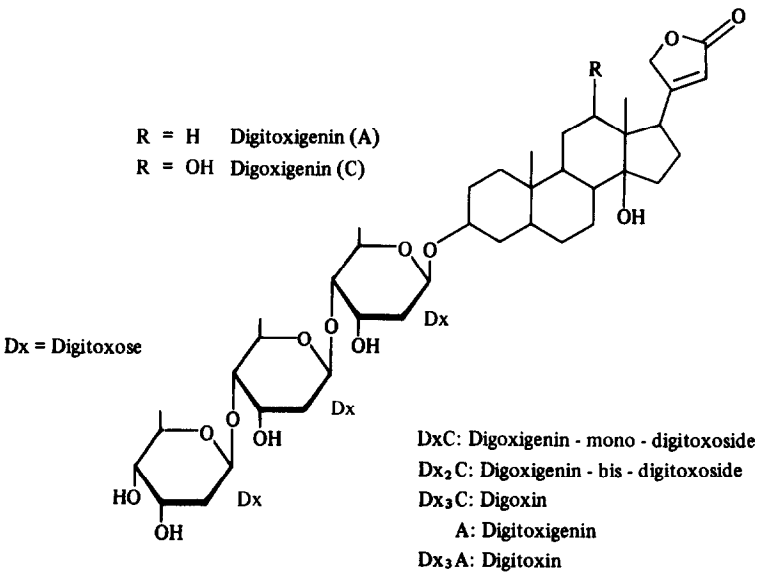

Fig. 1. Cardenolide structures.

biomass production above the values recorded at 350 ppm. Maximal weight and cardenolide content were achieved with additional CO_2 and adequate moisture (Table 1). This effect can be attributed to the higher CO_2 assimilation rate during the first (and vegetative) season of this biennial plant. The cardenolide content and both fresh and dry weight increased by about 60% at an elevated partial pressure of almost three times the natural concentration. As both dry weight and cardenolide content were equally affected, the proportions of carbon partitioning between primary 'growth' metabolism and secondary metabolism remained unchanged during CO_2 enrichment. The increased cardenolide yield per plant was directly connected with a more rapid growth.

Significant changes in individual components of the cardenolide spectrum were observed, although the total cardenolide content per dry weight was almost unaffected by the additional CO_2 (Table 2). On a mass basis, the digoxin content increased by about 10% in CO_2 enriched plants, whereas the amounts of digitoxigenin and digoxigenin-bis-digitoxoside were reduced. The increase in the main pharmaceutial product, digoxin, which ultimately accounted for more than 52% of the total cardenolides (Table 3), and the losses in other intermediates may be attributed to the occurrence of further biosynthetic activities in response to the faster development [8] of plants at higher atmospheric CO_2 levels.

Responses during water deficiency

The retardation of plant growth during water stress is a well known phenomenon [9]. Growth reduction was apparent in moderately stressed plants ($\psi = -1.3$ MPa) compared to well-watered controls ($\psi = -0.7$ MPa) (Table 1). Withholding irrigation resulted in severe decreases in fresh weight and slight reductions in carde-

Table 1. Fresh weight, dry weight and cardenolide content per plant in response to CO_2 enrichment and water deficiency treatment

CO_2 concentration	Plant parameters	Irrigated plants $\psi = -0.7$ MPa*	Stressed plants $\psi = -1.3$ MPa*
350 ppm	Fresh weight (g)	106.3	62.7
	Dry weight (g)	21.4	16.9
	Cardenolide content (μmol)	160.7	109.0
1000 ppm	Fresh weight (g)	171.9	90.0
	Dry weight (g)	34.9	30.9
	Cardenolide content (μmol)	260.2	199.4

Data presented are the means of either 8 replicates (fresh and dry weight) or 4 replicates (cardenolide content). Differences in all parameters are significant ($p < 0.05$).

*Water potential was measured at the youngest fully expanded leaf.

Table 2. Cardenolide content (nmol per g dry weight) in response to CO_2 enrichment and water deficiency

CO_2 concentration	Cardenolides†	Irrigated plants $\psi = -0.7$ MPa*	Stressed plants $\psi = -1.3$ MPa*
350 ppm	Total	7510	6440
	Digoxin-mono-digitoxoside	220	220
	Digoxin-bis-digitoxoside	1062	902
	Digoxin	3565	2965
	Digitoxigenin	1320	686
	Digitoxin	219	249
1000 ppm	Total	7450	6460
	Digoxin-mono-digitoxoside	337	278
	Digoxin-bis-digitoxoside	579	628
	Digoxin	3923	3401
	Digitoxigenin	760	258
	Digitoxin	290	225

Data presented are the means of either 4 replicates (total cardenolides) or 3 replicates (individual cardenolides). All differences in digoxin and digitoxigenin contents are significant ($p < 0.05$). The differences in digoxin-bis-digitoxoside are significant only for the CO_2- treatment, those in total cardenolides only for the water stress treatment.

*Water potential was measured at the youngest fully expanded leaf.

†Though not estimated quantitatively, traces of digitoxigenin-bis-digitoxoside, gitoxigenin, digoxin, neodigoxin and digoxigenin-tetra-digitoxoside could be detected in all samples.

Table 3. Relative cardenolide contents (per cent of total cardenolides) in response to CO_2 enrichment and water deficiency

CO_2 concentration	Cardenolides	Irrigated plants $\psi = -0.7$ MPa*	Stressed plants $\psi = -1.3$ MPa*
350 ppm	Digoxin-mono-digitoxoside	2.9	3.4
	Digoxin-bis-digitoxoside	14.1	14.0
	Digoxin	47.5	46.0
	Digitoxigenin	17.6	10.7
	Digitoxin	2.9	3.9
1000 ppm	Digoxin-mono-digitoxoside	4.5	4.3
	Digoxin-bis-digitoxoside	7.8	9.7
	Digoxin	52.7	52.6
	Digitoxigenin	10.2	4.0
	Digitoxin	3.9	3.5

The compounds quantified represent about 75% of the total cardenolides.

*Water potential was measured at the youngest fully expanded leaf.

nolide production and dry weight. Water loss of the plants preceded the metabolic changes. In the physiological stress range, where plants were able to recover completely, a decrease in the total cardenolide content per dry weight was apparent. These findings with the total amount of cardenolides extend Wurst's [5] earlier results, which showed an increase in lanatosides starting postmortally after a similar initial decrease.

Moderate water stress induced a considerable loss of digitoxigenin in relation to the other cardenolides, which were only slightly affected (Table 3). This may be due to the slow turnover of cardenolides [10] and a restricted flow of metabolites during water stress, which first affects the pool size of a primary product digitoxigenin. Additionally, stress could induce changes in the activities of the enzymes of secondary metabolism leading to the intensified use of alternative biosynthetic routes for glycoside synthesis [11, 12], maintaining relatively high levels of the other cardenolides. A moderate reduction in the major product, digoxin, and the substantial loss of

digitoxigenin accounted for approximately the observed decrease in the total cardenolide content (Table 2).

Responses by CO_2 enrichment and water deficiency

Withholding irrigation from plants grown in 1000 ppm CO_2 produced the combined effects of both treatments. Water deficiency affected the cardenolide content and the dry weight less in plants grown at 1000 ppm CO_2 than in those grown at 350 ppm (controls) (Table 1). The water stress effect on fresh weight, however, was more pronounced at elevated CO_2 than at ambient CO_2. Evidently, plants were able to utilize assimilated carbon much better during water stress and CO_2 enrichment than during water stress under natural conditions.

The cardenolide contents obtained from water stressed plants can simply be interpreted as the sum of the effects of additional CO_2 and water deficiency. Following CO_2 enrichment, the digoxin concentration in water stressed plants is significantly enhanced by comparison with plants which were subjected to an equivalent water stress at ambient CO_2 (Table 2). As in well-watered conditions digoxin-bis-digitoxoside and digitoxigenin contents decreased in CO_2 enriched atmosphere. In relation to well irrigated plants grown at 1000 ppm CO_2, it is obvious that water stress also contributed to the decrease in digitoxigenin (Table 3).

Our experimental data indicate that an increase in the CO_2 concentration of the surrounding air causes not only an increase in growth, but also an enhancement of secondary metabolites. In addition, the composition of the cardenolide spectrum is considerably altered, leading to an increased production of the pharmaceutically relevant digoxin.

Because of the gain in yield, artificial CO_2 enrichment promises the potential for improved cultivation of medicinal plants, such as *D. lanata*. Carbon dioxide enrichment could be applied during the early season of *Digitalis* cultivation, as long as the plants are greenhouse grown. Carbon dioxide fertilization can lead to an increased yield during the relatively short vegetative period in temperate climates. This is due to a more rapid growth which permits the accumulation of more dry matter and correspondingly more cardiac glycosides, in a given period of time.

In the context of the overall rising atmospheric CO_2 concentration, which is estimated to reach high levels in the next century [13], these results are also relevant. More plant material will be available to consumers, but the composition of the secondary metabolite spectrum, including the cardenolides, is also likely to be altered and correspondingly a change of the protective ability of the plant will occur.

In summary, variations in environmental conditions not only induce changes in primary production but also lead to complex alterations in secondary metabolism. The competitive performance of plants in an ecosystem may be influenced by these secondary metabolites.

EXPERIMENTAL

Digitalis lanata EHRH, a cultivar of the Boehringer Mannheim GmbH, had been parentally selected for the production of digoxin. The plants were grown for 130 days in a phytotron with 16 hr 250 μmol/m^2/sec photosynthetic active radiation at 24°, 75% relative humidity and 8 hr darkness at 17°, 75% relative humidity.

CO_2 enrichment in a separated chamber was achieved by adding CO_2 versus an external loop air stream, thus avoiding concentration gradients. The level of CO_2 was maintained at 1000 ± 35 ppm by continuously monitoring the chamber gases with an IR gas analyser. Threshold level contacts regulated the CO_2 valves.

Seeds were soaked for 3 days in light under running tap water and then germinated in garden soil (Einheitserde ED 73, Einheitserdenwerk Stangenberg, Hameln, Germany) mixed with 10% (v/v) sand.

After 4 weeks the plantlets were transferred to 11 cm diameter pots and after 10 weeks to 16 cm diameter pots. At each transfer the plantlets were selected for morphological similarity. Unstressed plants were watered daily with a commercial nutrient solution (Compo Gartendünger, Münster, Germany). Drought stress was induced 10 days before harvesting by withholding watering. Leaf water potential (ψ), measured according to ref. [14] in the youngest fully expanded leaf from above, was about -0.7 ± 0.05 MPa in the controls, while in the stressed plants it decreased to *ca* -1.3 ± 0.05 MPa.

Leaves dried at 50° were powdered. Complete deglucosylation was achieved after 24 hrs in 20% EtOH [15] followed by extraction with 80% MeOH. After filtration, the alcohols were removed under vacuum. Deacylation was carried out by adding conc NH_3 soln to avoid hydrolysis of the butenolide ring. Cardenolides were extracted with $CHCl_3$ and this extract was further purified by shaking with H_2O. After evaporation the substances were partitioned between 80% MeOH and cyclohexane [16]. After drying and redissolving the cardenolides in MeOH, the Baljet reaction, which does not discriminate between individual glycosides [17], was used to estimate the total cardenolides. HPLC separation (ISCO dialagrad 384, 40 min gradient 25–50% MeCN in H_2O, 0.8 ml/min, Waters μBONDAPAK C 18 column, detection at 220 nm) was carried out after adding Convallatoxin as an int. stand. (for similar procedures refer to [18, 19]). Cardenolide standards were obtained from Boehringer, Mannheim.

Acknowledgements—We thank Prof. G. C. Bate and Dr A. Lerch for helpful discussions.

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