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Polyamine Content and Ethylene Production of an Ozone-Resistant and Sensitive White Clover Clone

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Key words: *Trifolium repens* L. cv. Regal, NC-R, NC-S, ozone resistance, putrescine, spermidine, spermine, ethylene, plant age.

Summary

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Two clones of *Trifolium repens* L. Regal (NC-R and NC-S) with a different sensitivity for ozone were analyzed for their constitutive free polyamine concentration (putrescine, spermidine, spermine) and ethylene production. Plants of 5, 7 and 10 weeks old were fumigated for 3 consecutive days at 100 nl l^{-1} ozone during 3 h per day. The effect of ozone on the non-conjugated polyamines was determined during and after fumigation.

The polyamine concentration of the non-fumigated plants was dependent on plant age and clone. The youngest ozone-sensitive NC-S plants (5 weeks) contained 20 % less putrescine compared to the resistant NC-R clone, but this did not affect the total polyamine pool. The 5-weeks-old NC-S clone produced significantly more ethylene. As the plants grew older the total polyamine concentration of NC-S gradually decreased in comparison to NC-R. At 7 weeks both putrescine and spermidine were significantly decreased, at 10 weeks even the spermine content was significantly lower in NC-S compared to NC-R. Ozone had a significant effect on the total polyamine pool after 3 days exposure but the two clones did not respond in the same way. For the NC-R clone no significant change in polyamine concentration could be detected, whereas NC-S reacted with an increase in putrescine and spermidine. The ozone effect was dependent on plant age, being more pronounced in 7- and 10-weeks-old plants than in plants at 5 weeks of age.

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Introduction

The ozone-resistant NC-R and sensitive NC-S clones of *Trifolium repens* L. cv Regal are used worldwide to biomonitor the impact of ozone on plant biomass and visible injury (HEAGLE & al. 1995, MILLS & al. 2001). It has been shown that the NC-S/NS-R dry weight ratio diminishes as the ozone exposure increases (MILLS & al. 2001). The reason for this difference in ozone sensitivity is not yet completely understood and may have a physiological (stomatal conductance) or biochemical background (e.g. antioxidative enzyme activity), or a combination of both.

LANGEBARTELS & al. 1991 indicated that either polyamine or ethylene pathways may represent a control mechanism for inhition or promotion of lesion formation and thereby contribute to the disposition of the plants for ozone tolerance. The diamine putrescine, as well as spermidine and spermine, all commonly termed polyamines, exert several functions, which counteract ozone effects. They have been implicated in an inhibition of lipid peroxidation of membranes (LESTER 2000), an activation of membrane-bound ATPases (HEATH 1988), and a reduction of ethylene formation (SUTTLE 1981). It was further demonstrated that polyamine conjugates, but not the free polyamines, were effective scavengers for oxyradicals (BORS & al. 1989). Polyamines were observed to accumulate in e.g. O_3-treated barley (ROWLAND-BAMFORD & al. 1989), wheat (RAAB & WEINSTEIN 1990, AN & WANG 1997) and Aleppo pines (WELLBURN & al. 1996). Ethylene, which appears to be antagonistic to polyamines in senescence (SMITH 1990), is induced by O_3 in many plant species (WELLBURN & WELLBURN 1996).

The objective of the present study was to investigate whether free polyamine and ethylene metabolism was associated with the ozone tolerance of the white clover clones NC-S and NC-R. Since polyamine content is age dependant and may be involved in the control of plant senescence (BALLACH & al. 1995, FLORES 1990, GALSTON & SAWHNEY 1990), just as ozone effects are dependant on phenological development (VANDERMEIREN & al. 1995) and may induce earlier senescence, plant age was included as an experimental factor.

Material and Methods

The plant material was started from cuttings of the ozone-tolerant NC-R and ozone-sensitive NC-S clone of *Trifolium repens* L. cv Regal, supplied by A. Heagle from the North Carolina State University (Raleigh, US). The plants were cultivated in a 1/1 mixture of peat soil and vermiculite to which 1.7 g l^{-1} slow release fertilizer was added (3-4 months release Osmocote N-P-K 14/14/14). After two weeks the soil was inoculated with a Rhizobium slurry (20 ml per pot of a 2 g l^{-1} solution). Plants were cultivated and exposed to ozone in controlled environment chambers with a 15-9 h day-night regime. Daytime conditions were 23°C and 60-70 % humidity; night temperature was 17°C and 70-80 % humidity. Photosynthetic active radiation at plant level was approximately 300 µmol m^{-2} s^{-1}.

Three fumigation experiments were conducted with 5, 7 and 10-weeks-old plants. The ozone treatment consisted of a 3 days exposure to 100 nl l^{-1} O_3 during 3 h per day. The control plants were grown in charcoal filtered air. Ozone was produced from pure oxygen by electrical discharge (Fischer ozone generator, Germany) and added to charcoal filtered air.

For the polyamine-analysis 5-6 fully developed leaves were sampled from each plant (exp.1 n=12; exp 2 n=18; exp 3 n=8) within 2 hours after the end of fumigation (early afternoon) and frozen in liquid nitrogen. Leaves were sampled at random to obtain an idea of the average polyamine concentration of the entire plant, not just of one leaf at a specific stage. The samples were stored at –80°C. Putrescine, spermidine and spermine were extracted with perchloric acid and determined by HPLC-analysis (WALTER & GEUNS 1987). According to this method these polyamines can be referred to as free polyamines, and presumably are present inside the cell in ionized form at physiological pH (FLORES & PROTACIO 1990). Total polyamine content represents the sum of putrescine, spermidine and spermine. The constitutive polyamine concentration was determined on plants of the charcoal filtered treatment at 5 different occasions over a time span of two weeks before, during and after fumigation. These results were analyzed by ANOVA with repeated measures considering each individual plant as a subject measured over several days (Unistat 4.5, UK). To determine the ozone effect both fumigated and non-fumigated clover leaves were randomly sampled after one (day 1) and 3 days (day 3) of ozone fumigation, and 5 days after fumigation (day 8). The ozone effect was statistically analyzed by a 3-way ANOVA, including plant age and clone as additional factors.

Ethylene release was measured from leaf cuttings (diameter 12 mm) taken from 5-week-old control plants over a period of one week. Per sample 3 leaf disks were placed in a sealed glass vessel (10 ml) containing a sodium phosphate buffer solution (10 mM, pH 7.0) according to the method used by MEHLHORN & WELLBURN 1987. The vessel was stored for 24 h in the growth chamber and then analyzed by GLC (Carlo Erba series 6000 Vega) using flame ionization detection with He 99.99 % carrier gas flow rate of 18 cm s^{-1}. The results were statistically analyzed by repeated measures ANOVA.

Results

The total free polyamine concentration was dependent on clone and plant age with interaction between both factors (Table 1). In the 7- and 10-weeks-old plants the polyamine content of the NC-S clone was clearly decreased in comparison to the NC-R clone, whereas there was no significant difference in the 5-weeks-old plants (Fig. 1). The difference in total polyamine content between both clones was mainly caused by putrescine en spermidine (Table 1). The spermine content did not differ between both clones and was only dependant on plant age. The influence of plant age and clone became most evident by considering the ratios of the polyamine concentrations in NC-S over NC-R (Table 2). In 5-weeks-old plants the NC-S/NC-R ratio was 0.95, but in the 7-weeks-old plants the polyamine concentration of NC-S was reduced by 29 % compared to NC-R and in 10-weeks-old plants this reduction amounted to 37 %. So as the plants became older the polyamine concentration of the NC-S clone gradually decreased in comparison to the NC-R clone. The youngest plants showed a 20 % lower putrescine content in NC-S compared to NC-R, but due to the relatively smaller contribution of putrescine to the total polyamine pool this was not sufficient to have an effect on the total polyamine concentration. As plant age increased first spermidine and subsequently also spermine decreased more in NC-S than NC-R so that eventually also the total polyamine content was influenced.

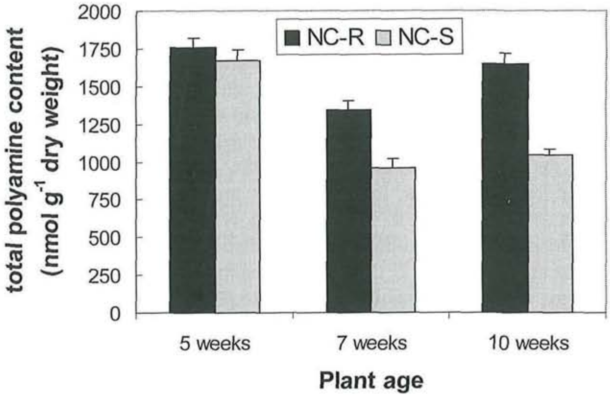

Fig. 1. Effect of plant age on the constitutive free polyamine concentration of *Trifolium repens* NC-R and NC-S clones. Vertical bars represent standard errors.

Table 1. Constitutive free polyamine concentration of *Trifolium repens* clones NC-R and NC-S averaged over 3 plant ages. The significance (p-value) of the clone and plant age effect is obtained by repeated measures ANOVA. SE, denotes standard error; ns, indicates no significant effect at the 5 % level.

	Mean (SE) ($nmol\ g^{-1}$ dry weight)		ANOVA (p-value)		
	NC-R	NC-S	Clone	Plant age	Clone x age
Total	1602 (43)	1263 (55)	<0.001	<0.001	0.002
Putrescine	445 (18)	286 (19)	<0.001	<0.001	0.010
Spermidine	739 (26)	571 (30)	<0.001	<0.001	0.013
Spermine	418 (20)	411 (21)	ns	0.004	ns

In the 5-weeks-old plants ethylene production was significantly ($p<0.001$) more elevated in the ozone-sensitive as compared to the tolerant clover clone (Table 3).

After 3 hours of ozone exposure on day 1 no ozone effects on total polyamine concentration were detected within the next two hours (Table 4, Fig. 2a); neither were there any significant changes with regard to the individual polyamines (data not shown). Only the age-dependant clonal differences in constitutive polyamine concentrations were still apparent, as previously discussed. Only after 3 days of fumigation ozone induced a significant change in total polyamine content that was dependent on plant age and clone (Table 4). The NC-S clone responded to ozone with a polyamine increase that was most pronounced in the 7- and 10-week old plants, which was not the case in the NC-R clone (Fig. 2b). Five days after fumigation the ozone effect was still significant but the clonal interactions were no longer apparent and the ozone effect depended highly on plant age (Table 4). Only the 7-week-old plants that had been previously exposed to ozone still showed an increased polyamine content compared to the controls, irrespective of the clone type (Fig. 2c). Due to the near significant (p=0.06) interaction between ozone, age and clone, it is however difficult to make any general statements with regard to the evolution of the polyamines after fumigation.

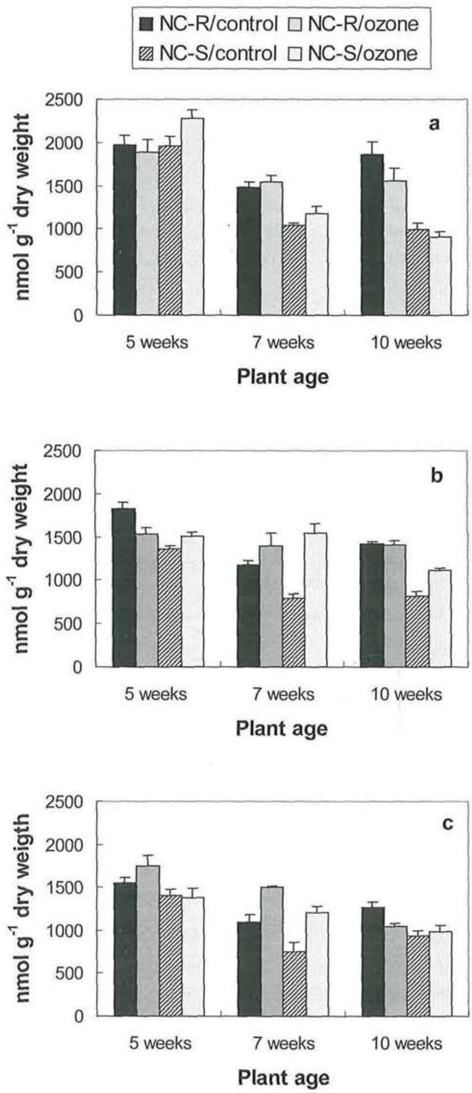

Fig. 2. Ozone effect on total free polyamine concentration of 5-, 7- and 10-week-old ozone-resistant NC-R and sensitive NC-S white clover clones a) after 1 day and b) after 3 days of ozone exposure, and c) 5 days after fumigation. Vertical bars represent standard errors.

Table 2. NC-S/NC-R ratio of the constitutive free polyamine concentration at 3 plant ages. The significance of the difference in polyamine concentration between the NC-S and NC-R clone is determined by ANOVA with repeated measures. *, ** and *** denote significance at the 5 %, 1 % and 0.1 % levels.

Plant age	Total polyamines	Putrescine	Spermidine	Spermine
5 weeks	0.95	0.80***	0.99	1.06
7 weeks	0.71***	0.47***	0.65***	1.05
10 weeks	0.63***	0.49***	0.60**	0.85*

Table 3. Average constitutive ethylene release from 5-weeks-old sensitive (NC-S) and resistant (NC-R) clover clones grown in charcoal filtered air, expressed on a projected leaf area basis. SE denotes standard error.

Clover clone	Mean ethylene production (SE)
NC-R	$2.11*10^{-2}$ ($\pm$ 0.31) ng h^{-1} cm^{-2}
NC-S	$7.10*10^{-2}$ ($\pm$ 0.59) ng h^{-1} cm^{-2}

Table 4. Statistical significance (p-value) of plant age, clone and ozone effects on total polyamine concentration after one day (day 1) and 3 days (day 3) exposure to 100 nl l^{-1} O_3 for 3 h day^{-1}, and 5 days after fumigation (day 8). ns denotes no significant difference at the 5 % level.

Effect	Day 1	Day 3	Day 8
Plant age	< 0.001	< 0.001	< 0.001
Clone	<0.001	< 0.001	< 0.001
Ozone	ns	< 0.001	0.003
Age x clone	<0.001	0.02	ns
Age x ozone	ns	<0.001	<0.001
Clone x ozone	ns	<0.001	ns
Age x clone x ozone	ns	Ns	ns

The age-dependent ozone effect that was observed on days 3 and 8 was caused by changes in all 3 polyamines, whereas the different clone response to ozone on day 3 was only significant for spermidine (Table 5). On day 3 the putrescine content of the 5-weeks-old plants remained unchanged after ozone exposure, but was increased in the 7- and 10-weeks-old NC-S clones. The effect on total polyamine content was mainly caused by spermidine, being the most abundant polyamine. In the resistant clone no consistent response to ozone was detected for either of the polyamines, whereas the sensitive clone showed a significant increase in spermidine after ozone exposure (Fig. 3b). Spermine response to ozone was highly dependent on plant age, but did not respond differently in both clones. Strangely the spermine content of 5- and 10-weeks-old plants decreased on the last day of exposure but increased in the 7-weeks-old plants (Fig. 3c).

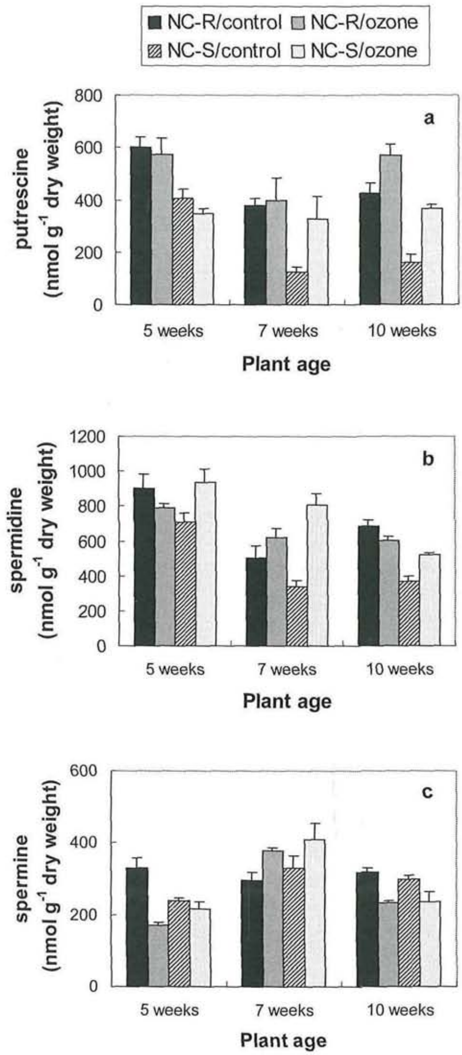

Fig. 3. Ozone effect on the a) putrescine, b) spermidine and c) spermine concentration of the ozone- sensitive (NC-S) and resistant (NC-R) white clover clones on the third day of fumigation. Vertical bars represent standard errors.

Table 5. Statistical significance (p-value) of plant age, clone and ozone effects on the putrescine (Put), spermidine (Spd) and spermine (Spm) concentration after 3 days exposure to 100 nl l^{-1} O_3 (day 3) and 5 days after fumigation (day 8). ns, denotes no significant difference at the 5 % level.

Effect	Day 3			Day 8		
	Put	Spd	Spm	Put	Spd	Spm
Plant age	<0.001	<0.001	<0.001	<0.001	<0.001	0.01
Clone	<0.001	0.03	ns	<0.001	<0.001	ns
Ozone	0.008	<0.001	0.04	ns	0.009	<0.001
Age x clone	ns	0.014	ns	ns	Ns	ns
Age x ozone	0.013	0.002	<0.001	0.02	<0.001	0.001
Clone x ozone	ns	<0.001	ns	ns	Ns	ns
Age x clone x ozone	ns	ns	ns	0.03	Ns	ns

Discussion

In this publication the polyamine content and ethylene release of an ozone-sensitive and resistant white clover clone was examined as a possible explanation for their difference in ozone tolerance.

At 5 weeks of age the constitutive total non-conjugated polyamine concentration of both clones was not significantly different, although the NC-R clones contained 20 % more putrescine. This agrees with the findings of YE & al. 1997 that constitutively elevated levels of putrescine and putrescine-generating enzymes may play an important role in contributing to the oxidant stress resistance. The equal spermidine and spermine content of both clones is rather contradictory to the higher ethylene release of the non-fumigated NC-S clone in comparison to NC-R. Ethylene formation will deviate part of the common precursor *S*-adenosylmethionine away from spermidine and spermine production. The functioning of this metabolic switch however may depend on the adenosylmethionine concentration (KUSHAD & al. 1988). If the pool is large enough both ethylene formation and polyamine biosynthesis can proceed unhindered (SMITH 1990).

As the plants become older the constitutive polyamine concentration of the non-fumigated sensitive NC-S clone decreases more rapidly compared to the resistant NC-R clone. In an ozone-sensitive poplar clone BALLACH & al. 1995 also observed a decreasing putrescine, spermidine and spermine content with increasing leaf age. FLORES 1990 reported that a decline in polyamine titers coincides with senescence-related symptoms. Our observation could thus be an indication that the ozone-sensitive clone proceeds to senescence at a quicker pace compared to the resistant clone, even under control conditions. In the absence of ozone we generally noticed a more abundant growth of the NC-S compared to NC-R plants. This may imply a faster exhaustion of the soil minerals inflicting nitrogen deficiency, which could consequently result in changes in polyamine concentrations. However, the applied slow release fertilization should have provided the plants with sufficient minerals during their entire life span.

Ozone fumigation at a concentration of 100 nl l^{-1} during 3 hours per day caused visible injury on the 5- and 7-weeks old NC-S clone during the third day; 10-weeks-old plants already showed ozone symptoms after one day of fumigation. Differences in ozone uptake did not appear to lie at the origin of the different ozone tolerance of both clones. Stomatal conductance measured at the start of fumigation did not indicate a significant difference between the NC-R and NC-S clone (unpublished data).

Ozone exposure did not significantly affect the total free polyamine concentration of the resistant clone. After 3 days ozone induced a polyamine increase in the older NC-S plants (7 and 10 weeks). It is mainly the spermidine concentration that is most significantly increased in NC-S; even though putrescine was also more strongly increased in 7- and 10-weeks old NC-S plants. Since these plants also showed visible injury at that time, whereas the 5-weeks-old plants did not, it seems most likely that the polyamine increase is a consequence of cell damage rather than a protective mechanism against oxidative stress. FLORES 1990 hypothesized that putrescine accumulation is a toxic response resulting from ionic stress and may be deleterious to the plant cell. Putrescine increase may be caused by inactivation of extracellular diamine oxidase by O_3 or H_2O_2 (PETERS & al. 1988) or increased activation of arginine decarboxylase (LANGEBARTELS & al. 1991). Spermidine increase is most probably a consequence of the increased availability of its substrate putrescine.

Although the differences in constitutive free polyamine concentration and their response to ozone do not provide an unequivocal explanation of the difference in ozone sensitivity between both white clover clones, they do reveal a correlation between the polyamine metabolism and ozone tolerance. This interaction is a dynamic process that is significantly influenced by plant age.

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