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# Developmental processes, polyamine composition and content of fruiting cuttings of *Vitis vinifera* L.: Responses to nitrogen deficiency

L. Geny[1] and M. Broquedis[2]

[1] Laboratoire de Pédologie, Physiologie de la Vigne, Faculté d'Oenologie, Université Bordeaux II Victor Ségalen, Talence, France
[2] Laboratoire des Sciences de la Vigne, Université Bordeaux I, Talence, France

## Summary

**Fruiting cuttings of cv. Cabernet Sauvignon exposed to nitrogen (N) deficiency showed alterations of the N and phosphorus content, the number of berries per plant and berry characteristics at maturity. Conjugated and bound polyamines changed in various tissues at different and critical stages of development and responded strongly to N deficiency. At anthesis the conjugated and bound spermidine content in flowers and the N supply were closely and inversely correlated, while conjugated and bound forms of putrescine responded in the opposite way. However, in these organs N deficiency led to a high content of bound spermidine and a marked decrease of conjugated putrescine. At fruit set, N deficiency was associated with a high content of bound diaminopropane in berries. In ripe berries free putrescine and the N supply were inversely correlated.**

K e y w o r d s : *Vitis vinifera* L., polyamines, conjugated polyamines, bound polyamines, fruiting cuttings, nitrogen deficiency.

## Introduction

Polyamines are small, positively charged aliphatic amines that play various roles in plant physiology, including the regulation of DNA replication, transcription of genes, cell division, organ development, floral process, fruit ripening and leaf senescence (Egea-Cortines *et al*. 1991). In higher plants, the polyamine metabolism responds to external conditions especially to mineral nutrient deficiencies (Flores *et al*. 1985) and is also involved in plant responses to microbial symbionts (Ghatchtouli *et al*. 1996). Furthermore, as a result of potassium deficiency putrescine accumulates in mono- and dicotyledonous species and may well be a universal response (Flores 1991). In all cases, maximum putrescine accumulation coincided with the appearance of severe nutrient deficiency symptoms (Flores 1991). It has been reported that in continuously dividing plant cells of *Medicago varia,* nitrogen deficiency led to an arrest of the cell cycle and to a depletion of free polyamine levels (Pfosser *et al*. 1992). Nitrogen has no deleterious effects on cell viability. Withdrawal of nitrogen allowed most of the cells to undergo a few rounds of replication prior to the arrest of the cell cycle, polyamine titers being closely linked to decreasing cell division. The transition of cells to the quiescent state is possibly supported by the use of degraded polyamines as a nitrogen source (Pfosser *et al*. 1992).

In higher plants attention has focused mostly on free polyamine responses to mineral nutrient deficiencies. Most earlier studies did not analyse polyamine catabolism and the conjugated and bound forms of polyamines. Conjugated polyamines are covalently linked to hydroxycinnamic acids. Bound polyamines are linked to macromolecules, such as proteins and are correlated with several developmental phenomena (Martin-tanguy 1997). Conjugated forms have important functions in floral induction, reproduction and fruit ripening; *e.g*., the availability of conjugated spermidine is a limiting factor in sexual development in tobacco (Martin-tanguy 1997). It has to stressed that most studies on polyamines related to stress responses have been done only in leaves and/or in seedlings, when the symptoms of deficiency became visible.

The objective of the present study was to determine the effects of N deficiency on the development of fruiting cuttings of *Vitis vinifera* L. and on the composition and the content of free, conjugated and bound forms of polyamines in various tissues at different and critical stages of development.

## Material and Methods

P l a n t m a t e r i a l a n d c u l t u r e c o n d i t i o n s : Fruiting cuttings were cultivated according to Mullins and Rajasekaran (1981). Dormant cuttings from one-year-old, cane-pruned Cabernet Sauvignon vines were collected in a vineyard at Blanquefort (Bordeaux, France). The cuttings were propagated by ensuring that the formation of adventitious roots preceded bud burst (26 °C at the base of the cuttings, 4 °C room temperature). After 4 weeks, the pre-rooted cuttings were planted in pots (100 $cm^3$) containing a mixture of Perlite/sand, and were transferred to a chamber with controlled environment (27/22 °C day/night, 70 % RH, photoperiod 14 to 16 h). Irradiance (300 µmol $m^{-2}$ $s^{-1}$ PPFD) was provided by Philipps TLF incandescent lamps and 40 W incandescent lamps). The hydroponic solution was provided by drip irrigation (150 ml·$day^{-1}$·$pot^{-1}$). Experimental media were a N-deficient medium (-N) and a control (Tab. 1).

S a m p l i n g p r o c e d u r e : Samples were taken from fruiting cuttings for each treatment at anthesis, berry set, before veraison ('unripe') and at berry maturity. The samples, flowers, unripe and ripe berries (pulp and skin) and apical leaves, were taken on the some day. Leaves picked at fruit ripening did not show any symptoms of N deficiency or senescence.

Correspondence to: Dr. L. Geny, Faculté d'Oenologie, Université Victor Ségalen Bordeaux 2, 351, cours de la Libération, 33405 Talence Cedex, France. Fax: +33-5-5684-6468. E-mail: oenoadm@oenologie.u-bordeaux2.fr

Table 1

Concentration of macronutrients in the hydroponic solution (mmol $l^{-1}$)

| Conditions | N | P | K | Ca | Mg | S |
|---|---|---|---|---|---|---|
| Control | 7.16 | 0.57 | 1.27 | 0.95 | 1.02 | 1.01 |
| -N | 1.91 | 0.57 | 1.27 | 0.95 | 1.02 | 1.36 |

Cation analyses: Samples were dried at 80 °C to constant weight and digested with $H_2SO_4$ plus $H_2O_2$. Cations (N, P) were analysed by inductively coupled plasma spectrometry according to TURNER and BROOKS (1992).

Polyamine analysis: Samples (100 mg fresh weight) were homogenized according to FLORES and GALSTON (1982). After extraction (1 h) in an ice bath, samples were pelleted at 48,000 *g* for 20 min and the supernatant phase containing the free polyamine fraction, was stored at -20 °C.

High Performance Liquid Chromatography and fluorescence spectrophotometry were used to separate and quantify polyamines prepared as dansyl derivatives, according to SMITH and DAVIES (1985). A column (4x240 mm) with reversed-phase Purospher LP18/Merck, particle size 5 µm, was used. Samples were eluted with a programmed acetonitrile/water (v/v) solvent gradient, changing from 35 to 65 % in 8 min at a flow rate of 1 ml $min^{-1}$. Elution was completed with 65 % acetonitrile in 17 min. The column was washed with 100 % methanol for 5 min and reequilibrated with 35 % acetonitrile in 25 min before the next sample was injected.

Polyamines and eluates from the column were detected by a fluorescence spectrophotometer with a 5µl flow-through cell (model LC4000, TSP). For dansyl polyamines, an excitation wavelength of 365 nm was used with an emission wavelength of 510 nm. Peak area and retention time were recorded by an attached integrator.

For quantification of conjugated polyamines, supernatants were boiled in 6 M HCl followed by determination of amines (SLOCUM and GALSTON, 1985). The amounts of conjugated polyamines were calculated as the difference between total and free levels. Bound polyamines were estimated from pellet fractions as reported previously by SLOCUM and GALSTON (1985). Conjugated polyamines were identified according to PONCHET *et al*. (1980) and MARTIN-TANGUY *et al*. (1978).

Experimental repetition and statistical analysis: Analytical data are means ± SD of two experiments, each experiment consists of 5 fruiting cuttings. Data concerning effects of N deficiency on grapevine development are means ± SD of two experiments, each experiment consisting of 40 fruiting cuttings.

## Results

Effects of nitrogen deficiency: As a symptom of nitrogen ($N_2$) deficiency a lowering of chlorophyll became visible in basal leaves in the stage of fruit ripening. Moreover, the leaves became necrotic and showed reddish margins. These symptoms progressed from bottom to top leaves.

In response to N deficiency, the number of berries per plant was reduced by approximately 25 % while the berry and seed fresh weight at maturity had increased by about 30 and 60 %, respectively (Tab. 2). The number of seeds per berry was not significantly modified. N deficiency reduced the alcohol content by 26 %, whereas total acidity was increased about 1.5 times.

Table 2

Effects of N deficiency on berries and must quality at maturity. Means ± SD of two experiments (each consisted of 30 berries from 5 fruiting cuttings)

| | Control | -N |
|---|---|---|
| Berries formed (%) | 25 ± 4.0 | 19.0 ± 4.0 |
| Fresh weight of 100 berries (g) | 76.5 ± 2.2 | 106.3 ± 2.2 |
| Fresh weight of 100 seeds (g) | 3.1 ± 0.6 | 7.4 ± 0.6 |
| Number of seeds per berry | 1.5 ± 0.1 | 1.7 ± 0.1 |
| pH in must | 3.7 ± 0.3 | 3.3 ± 0.3 |
| Total acidity (g $l^{-1}H_2SO_4$) | 5.9 ± 0.8 | 8.9 ± 0.8 |

Irrespective of the N supply the N concentration in flowers, berries and leaves decreased from flowering until fruit ripening (Fig. 1 A). N concentrations were reduced in all organs at different stages of development (Fig. 1 A).

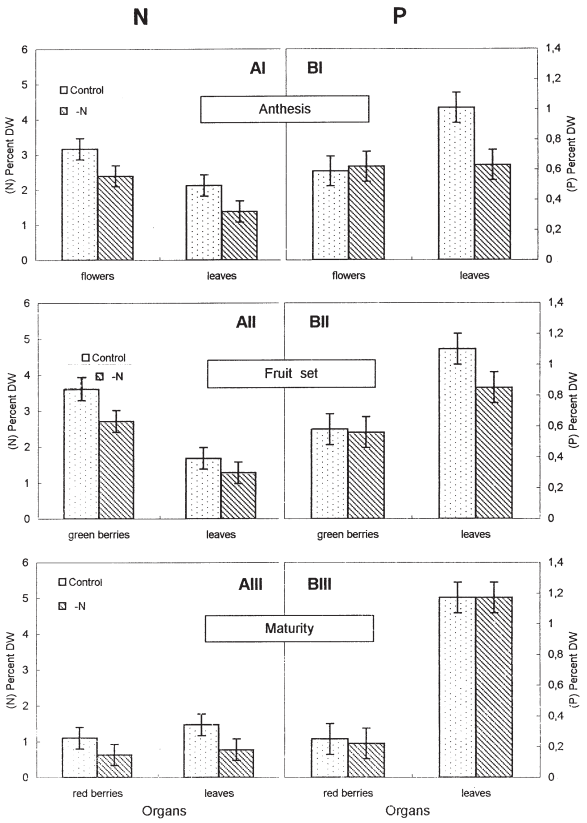


Fig. 1: Effects of nitrogen (N) deficiency on the N (left) and P (right) content in flowers at anthesis, before veraison ('green berries'), at maturity ('red berries') and in leaves of fruiting cuttings (Cabernet Sauvignon) at different stages of development. Means ± SD of two experiments, each experiment consisting of 500 flowers, 30 unripe berries or 30 ripe berries and 10 leaves from 5 fruiting cuttings.

Under both, normal and N deficient conditions (except for leaves), P concentration decreased from flowering until fruit ripening (Fig. 1 B). A decrease of the N/P ratio (except for leaves at anthesis and leaves during fruit set) was observed in organs of fruiting cuttings at different stages of development under N deficiency (Tab. 3). The lowest ratio was always observed in N deficient leaves in the stage of fruit ripening.

Table 3

Effects of N deficiency on the N/P ratio in flowers, berries and leaves of fruiting cuttings of cv. Cabernet Sauvignon at different stages of development. Means of two experiments, each consisting of 500 flowers, 30 unripe berries or 30 ripe berries and 10 apical leaves from 5 fruiting cuttings

| Stage of development | Organs | N/P | |
|---|---|---|---|
| | | Control | -N |
| Anthesis | flowers | 5.33 | 3.85 |
| | leaves | 2.09 | 2.18 |
| Fruit set | unripe berries | 6.14 | 4.86 |
| | leaves | 1.52 | 1.50 |
| Maturity | ripe berries | 4.49 | 2.84 |
| | leaves | 1.30 | 0.51 |

Effects of N supply on polyamines: The main free polyamines detected were putrescine (PUT), spermidine (SPD) and diaminopropane (DAP). In all organs spermine (SPM) remained at a low level. Conjugated polyamines contained hydroxycinnamic acids (p-coumaric acid at anthesis, ferulic acid at fruit set) and polyamines linked by an amide bound. Bound polyamines were PUT, SPD and DAP. The polyamine content and its composition differed between tissues and stages of berry development (Figs 2 A, 3 A and 4 A).

Anthesis: In flowers of N deficient plants, free PUT levels increased 3.5 times (Fig. 2 BI compared to Fig. 2 AI) while DAP, SPD and SPM were not significantly altered; conjugated SPD increased 3-fold (Fig. 2 BII compared to Fig. 2 AII). Levels of conjugated PUT and DAP decreased by about 70 and 60 % respectively. The absence of N slightly enhanced the concentration of conjugated SPM. In these tissues bound PUT decreased by about 47 % whereas bound SPD increased 1.7 times (Fig. 2 BIII compared to Fig. 2 AIII). Bound DAP remained relatively unaffected. At this stage of development bound SPD was the predominant polyamine, representing about 40 % of the total polyamine pool.

In leaves, N supply had no significant effect on free, conjugated and bound polyamines for which decreased 85 %) which remained at a low concentration (exception: conjugated PUT; Fig. 2 B compared to Fig. 2 A).

Fruit set: In unripe berries, nitrogen supply had no significant effect on the free polyamine content which remained at a low concentration (Fig. 3 BI compared to Fig. 3 AI), while PUT and DAP conjugate levels decreased by 67 and 85 %, respectively (Fig. 3 BII compared to Fig. 3 AII). N deficiency raised bound polyamine contents: PUT 1.5 times, SPD 3.2 times, SPM 2.7 times and DAP 9 times

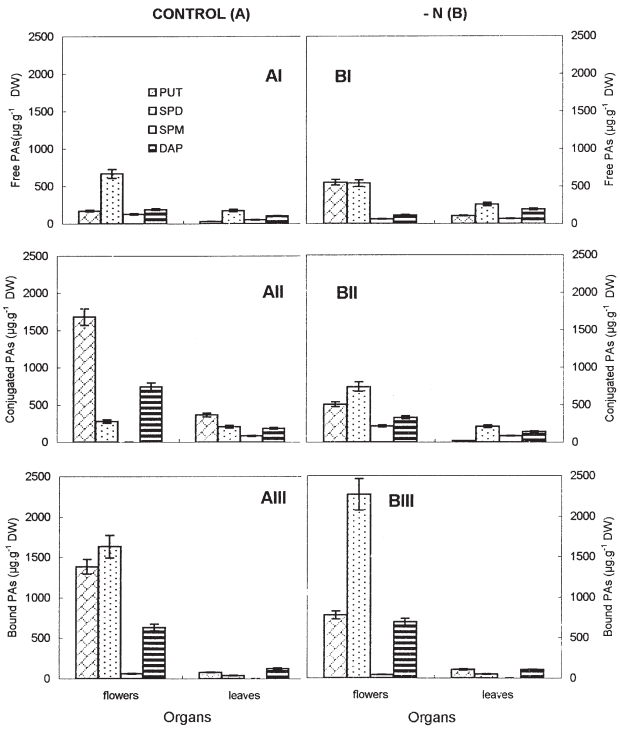


Fig. 2: Influence of nitrogen (N) deficiency on levels of polyamines (PAs) in flowers and leaves of fruiting cuttings of Cabernet Sauvignon at anthesis. **A**: control plants; **B**: N deficient plants. **I**: free PAs, **II**: conjugated PAs, **III**: bound PAs. Means ± SD of two experiments, each consisting of 500 flowers and 10 leaves from 5 fruiting cuttings.

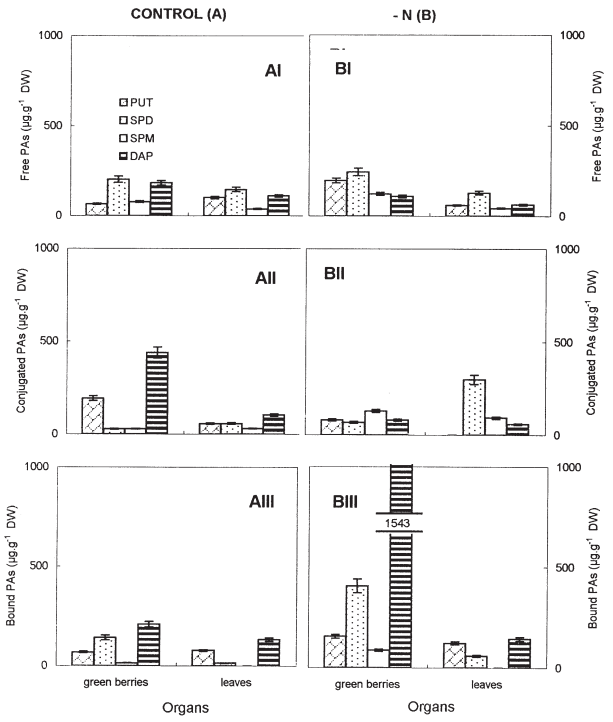


Fig. 3: Influence of nitrogen (N) deficiency on levels of polyamines (PAs) in unripe berries ('green berries') and leaves of fruiting cuttings (Cabernet Sauvignon) at fruit set. **A**: control plants; **B**: N deficient plants. **I**: free PAs, **II**: conjugated PAs, **III**: bound PAs. Means ± SD of two experiments, each consisting of 30 unripe berries and 10 leaves from 5 fruiting cuttings.

(Fig. 3 BIII compared to Fig. 3 AIII). At this stage of development, bound DAP was the predominant polyamine, representing 52 % of the total pool.

In leaves, PA levels did not exhibit major changes without N supply (except for conjugated SPD which increased 3 times). Polyamines remained at a low concentration (Fig. 3 B compared to Fig. 3 A) irrespective of the N supply.

Fruit ripening: In berries of N deficient plants, the free PUT content increased 1.7 times (Fig. 4 BI compared to Fig. 4 AI), while no PUT conjugates were produced (Fig. 4 BII compared to Fig. 4 AII). PUT, SPD and DAP slightly increased in berries (Fig. 4 BIII compared to Fig. 4 AIII).

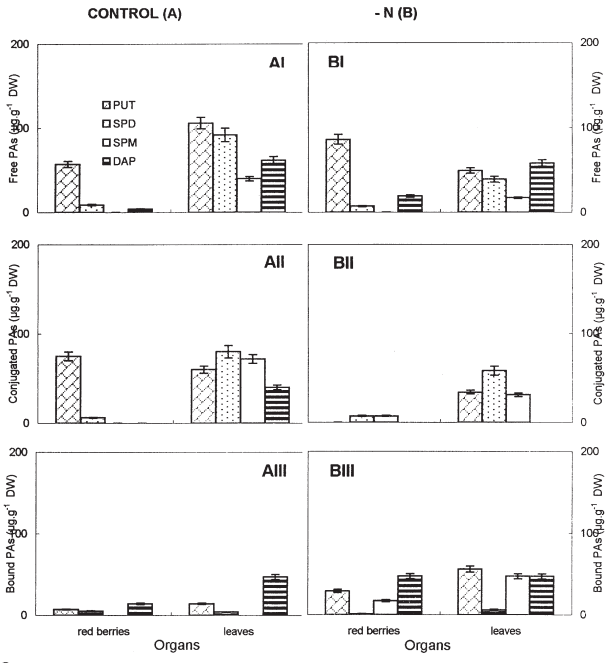


Fig. 4: Influence of nitrogen (N) deficiency on levels of polyamines (PAs) in ripe berries ('red berries') and leaves of fruiting cuttings (Cabernet Sauvignon) at maturity. **A**: control plants; **B**: N deficient plants; **I**: free PAs, **II**: conjugated PAs, **III**: bound PAs. Means ± SD of duplicate determination (one sample consists of 15 ripe berries and 10 leaves from 5 fruiting cuttings.

In leaves of N deficient plants, free and conjugated polyamine contents decreased whereas bound PUT and SPM increased. For free and wall-bound DAP contents the N supply had no significant effect (Fig. 4 B compared to Fig. 4 A).

## Discussion

Changes in the metabolism of free and conjugated polyamines during flowering and fruit development were similar to those reported for some other species (MARTIN-TANGUY 1997). However, to our knowledge, this is the first detailed report on the occurrence of bound polyamines in flowers and reproductive organs. Furthermore, conjugated and bound diaminopropane appear to be involved in the fruit set process.

At anthesis the conjugated and bound spermidine content in flowers was inversely correlated with the N supply. In contrast, in these organs, a positive correlation was observed with conjugated and bound forms of putrescine. These results reflect a different pool size of polyamines in floral tissues of control and N deficient plants, and suggest that putrescine was metabolized faster in the N deficient plants. This was probably due to an increased conversion of putrescine to spermidine. The results support the view that the increased level of bound spermidine might result from a metabolic modification caused by N deficiency. At this stage of development, N deficiency induced a complete depletion of conjugated putrescine.

At fruit set, the most significant effect of N deficiency was a high content of bound diaminopropane in berries. These results suggest that N deficiency increases the binding of diaminopropane to insoluble cell components.

These data raise the question if diaminopropane plays a role in nitrogen deficient tissues. SHIH *et al.* (1982) demonstrated that diaminopropane is a potent inhibitor of senescence. Under our experimental conditions this compound may have delayed the onset and progress of senescence. On the other hand, polyamine oxidase activity produces $H_2O_2$ as a reaction product; it has been suggested that this activity might influence growth *via* coupled $H_2O_2$ dependent peroxidase-mediated cross-linking of cell wall polymers such as lignin (ANGELINI and FREDERICO 1989). At this stage of development a complete depletion of conjugated putrescine and some increase of conjugated spermidine occurred in leaves of nitrogen deficient plants.

In contrast to conjugated putrescine in ripe berries there was an inverse correlation between free putrescine and the N supply. In mung bean, the ratio of bound to free polyamines was high during the growth stages and decreased during senescence (GOLDBERG and PERDRIZET 1984). Some reports demonstrated a high level of endogenous binding of putrescine and especially spermidine to proteins in actively dividing young tissues and a lower binding in mature non-dividing tissues (APELBAUM *et al.* 1988; ARIBAUD *et al.* 1995). A high level of bound polyamines in N deficient organs may play a role in the maintenance of cell division and membrane integrity. It has been proposed that the conjugates may be involved in polyamine translocation (HAVELANGE *et al.* 1996). These compounds are the preferred substrates for amine oxidases (MARTIN-TANGUY 1997) and they are substrates for peroxidases in cells of tobacco leaves (NEGREL and LHERMINIER 1987). Peroxydases which utilise putrescine conjugates can remove $H_2O_2$ in the apoplast. Conjugation of polyamines may regulate the interactions with inorganic cations such as $Ca^{2+}$, which might have an implication for the proposed role of PAs on membrane stabilization. Conjugation reactions could regulate polyamine functions by, *e.g.* affecting their binding and interaction with nucleic acids and proteins or phospholipids. Moreover polyamine conjugation to a hydroxycinnamoyl might be important for the regulation of the free polyamine titers and/or in detoxicating phenolic compounds known to inhibit growth (MARTIN-TANGUY 1997). It was also proposed that the conversion of polyamines to their conjugated forms is a prerequisite for their protective role in the presence of superoxides (FLORES 1991). Thus a decrease of polyamine conjugation may have negative effect on growth and development.

## Conclusion

This paper demonstrates that levels of conjugated polyamines respond to the N supply and changed during plant development. Causal effects in regulating growth and development may be found by cloning genes encoding for transglutaminases in fruiting cuttings of grape which will enable studies of the mechanisms controlling polyamine conjugation to proteins as well as to establish the underlaying molecular mechanisms.

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