J Plant Growth Regul (1989) 8:143–151

Journal of
Plant
Growth
Regulation

Nitroguanidines Induce Bud Break and Change Sterol Content in Apple

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Received July 22, 1988; accepted October 11, 1988

Abstract. Bud break in apple (*Malus domestica* Borkh cv. Golden Delicious) was induced by 1-(3,5-dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine. The optimum dose was 1000 μM. An increase in bud fresh weight, dry weight, and length was more prominent in buds treated with 1-(α-ethylbenzyl)-3-nitroguanidine than in those treated with 1-(3,5-dichlorophenyl)-3-nitroguanidine. The sterol compositional changes during bud break induced by 1-(3,5-dichlorophenyl)-3-nitroguanidine were similar to those induced by 1-(α-ethylbenzyl)-3-nitroguanidine. β-Sitosterol and sitosteryl ester were the predominant sterols. The amounts of these sterols increased immediately after dormancy was broken and then declined. A decrease in the percentage of the sitosterol and sitosteryl ester was accompanied by an increase in campesterol and stigmasterol at the beginning of rapid growth. A decrease in the ratio of free sterols to phospholipids and an increase in the ratio of campesterol + stigmasterol to sitosterol upon breaking dormancy occurred in apple buds induced by 1-(3,5,-dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine. 1-(m-Methoxybenzyl)-3-nitroguanidine did not affect breaking of apple bud dormancy and also had no effect on changes in sterol content. The sterols in apple buds were confirmed by gas chromatography–mass spectrometry.

Manipulation of plant growth, development, and metabolism with plant hormones and synthetic chemicals is important in agriculture (Hardy 1979,

Abbreviations: BHT, 2,6-di-*t*-butyl-4-methyl phenol; DMSO, dimethyl sulfoxide; EI, electron impact; FID, flame ionization detector; GC, gas-liquid chromatography; PAR, photosynthetically active radiation.

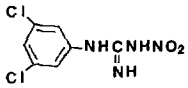
Bruinsma 1982). Dormant apple buds require a certain amount of low temperature before resumption of growth in the spring. Certain plant hormones and growth regulators can substitute for chilling to induce bud break. This permits the growing of deciduous fruit trees in warm areas where otherwise it would be impossible (Erez 1987). Sterols play a vital role during growth and development of higher plants (Garg and Paleg 1986). Sterol content and composition are related to membrane permeability and development in higher plants (Goad 1983, Grunwald 1975). Sterols may also serve as precursors to steroid hormones in plants (Heftmann 1971).

The plant bioregulant [N-phenyl-N′-1,2,3-thidiazol-5-ylurea (thidiazuron; Dropp; SN49537)] releases lateral buds from dormancy in apple (Wang et al. 1986). A decrease in the percentage of the sitosterol was accompanied by an increase in campesterol and stigmasterol at the beginning of rapid growth upon breaking of dormancy. An increase in the ratio of campesterol plus stigmasterol to sitosterol and a decrease in the ratio of free sterols to membrane lipids upon breaking of dormancy was also found in apple buds induced by thidiazuron (Wang and Faust 1988). Recently, a new class of synthetic cytokinins known as benzylnitroguanidines (Fig. 1) was found to elicit cytokinin activity in bioassay systems and to promote growth in cytokinin-dependent soybean callus cultures (Rodaway and Lutz 1985). These compounds also increase the rate of chlorophyll biosynthesis in dark-grown cucumber cotyledons, increase the expansion of radish cotyledons and lima bean leaf disks, and delay the loss of chlorophyll in excised, senescing potato leaves (Rodaway and Lutz 1985). Nitroguanidines are also sensitive to 7-(isopentylamino)-3-methyl-1H-pyrazolo-(4-3-d) pyrimidine, a known antagonist of adenine cytokinins (Rodaway and Lutz 1985). The present study was initiated to determine the effect of nitroguanidines on breaking bud dormancy and bud growth in apple seedlings. The changes of sterol content and the ratio of free sterols to phospholipids occurring concomitantly with breaking of bud dormancy by nitroguanidines were also examined.

Materials and Methods

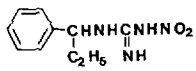
Plant Material and Treatments

The growing conditions and treatments of apple seedlings (*Malus domestica* Borkh. cv. York Imperial) used in this study have been reported (Wang et al. 1986). 1-(3,5-Dichlorophenyl)-3-nitroguanidine, 1-(α-ethylbenzyl)-3-nitroguanidine, and 1-(m-methoxybenzyl)-3-nitroguanidine (Fig. 1) were obtained from American Cyanamid Company (Princeton, NJ) and dissolved in 2.5% (v/v) DMSO plus 0.5% (v/v) Tween-20 and applied directly to the buds with a brush until runoff. Only the first five buds were treated. Radiation sources in the greenhouse consisted of natural daylight and 400-W high-pressure sodium lamps (Energy Technics, York, PA), which provided a PAR level of about 400–500 μmol m^{-2} s^{-1} for 12 h/day (0700–1900 h). Temperatures were about 25°C during the day and 20°C at night.



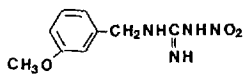
1-(3,5-dichlorophenyl)-3-nitroguanidine

(a)



1-(α-ethylbenzyl)-3-nitroguanidine

(b)



1-(m-methoxybenzyl)-3-nitroguanidine

(c)

Fig. 1. Chemical structures of three nitroguanidines.

Extraction, Fractionation, and Analysis of Lipids

Triplicate bud samples of 0.5 g fresh weight were collected at 5-day intervals over a 25-day period after treating with nitroguanidines. Buds were extracted with 10 ml isopropanol [containing 4 μg BHT $(ml)^{-1}$]. Nonlipid contaminants were removed as previously described (Folch et al. 1957). The purified lipids were separated on a silicic acid column (100- to 200-mesh BioSil A, BioRad Laboratories, Richmond, CA, USA) into neutral, glycolipid, and phospholipid. Total phospholipid was determined by a spectrophotometric assay (Ames 1966).

Free sterols and steryl esters in the neutral lipid fraction from apple buds were separated by TLC on silica gel plates (250 μm thickness, Merck G 60). Known amounts of cholesterol and cholesteryl myristate were included in all samples as internal standards. The plate was developed in a freshly made solvent mix of hexane/ethyl acetate/formic acid (80:20:2, v/v). Spots corresponding to free sterols and steryl esters, identified by spraying with $FeCl_3$ reagent (Christie 1973, Lowry 1968), were removed and eluted with 3 ml chloroform/methanol mixture 2:1 (v/v) [plus 4 μg BHT $(ml)^{-1}$]. After addition of 1 ml 0.8% NaCl, chloroform containing the free sterols or steryl esters was evaporated to dryness under N_2. Free sterols were resuspended in isooctane before GC analysis. Steryl esters were saponified in 1 ml of 1 M KOH in 85% ethanol for 1 h at 80°C (under N_2). After heating, 0.5 ml H_2O was added and the free

sterols were recovered by extraction with 2 ml of hexane. Samples were evaporated to dryness under N_2 and redissolved in 50 μl 100% ethanol for GC analysis. Sterol composition was determined by FID-GC (Wang and Faust 1988).

A Pye Unicam model 104 gas chromatograph equipped with a 0.9 m × 2.0 mm (i.d.) 3% OV 3 packed glass column and Kratos AEI MS-30 double beam mass spectrometer were used to confirm the identity of sterols. Column temperature was 260°C, isothermal. Ultrapure helium was used as carrier gas at a flow rate of 40 ml/min. The injector and jet separator temperature was 270°C. The mass range from 200 to 600 amu was scanned magnetically using dual beams in the EI mode. The mass number (EIMS, ionizing energy 70 eV) determined for each GC peak was compared with the mass measurement of authentic sterols.

Results and Discussion

Effect of Benzylnitroguanidines on Bud Break and Bud Growth

1-(3,5-Dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine stimulated bud break at concentrations ranging from 100 to 1000 μM (Figs. 2–4). The optimum dosage was 1000 μM. Higher concentrations were less stimulatory for releasing dormancy and inducing subsequent growth of buds (data not shown). Three days after treatment with 250–1000 μM of 1-(3,5-dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine, buds started to swell. Fresh weight, dry weight, and length increased more in buds treated with 1-(α-ethylbenzyl)-3-nitroguanidine than in those treated with 1-(3,5-dichlorophenyl)-3-nitroguanidine (Figs. 2–4). However, both compounds were 10–20 times less effective (based on the concentration of active agent) than thidiazuron in breaking dormancy in apple (data not shown). The untreated buds from the same plants and buds from control plants remained dormant (Figs. 2–4), indicating that nitroguanidine was not translocated in apple stems. The application of 1-(m-methoxybenzyl)-3-nitroguanidine had no effect in breaking apple bud dormancy at all concentrations (100–1000 μM) applied.

Change in Sterols

The sterol composition of apple buds was determined at 5-day intervals over a 25-day period after nitroguanidine treatment. β-Sitosterol, campesterol, stigmasteryl, and sitosterol ester were found in apple buds (Figs. 5, 6). These three phytosterols are typical plant sterol species (Grunwald 1975). The identity of these sterols was established by GLC and by mass spectrometry. The GLC-MS relative retention times of campesterol (9.17 min), stigmasterol (10.10 min), and sitosterol (11.35 min) were identical to those of authentic samples. The fragmentation patterns, significant ions, and relative intensities of the full EIMS (data not shown) matched the authentic standards, as previously reported (Heller and Milne 1978).

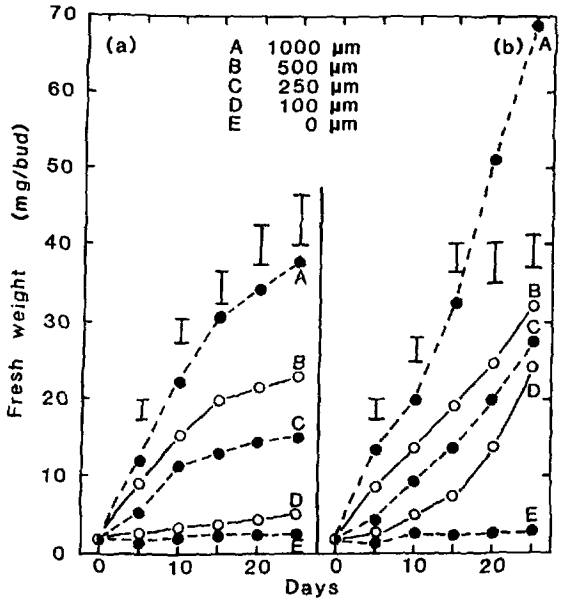


Fig. 2. Changes in fresh weight of apple buds induced by (a) 1-(3,5-dichlorophenyl)-3-nitroguanidine or (b) 1-(α-ethylbenzyl)-3-nitroguanidine. Vertical bar is LSD (5%) among treatments.

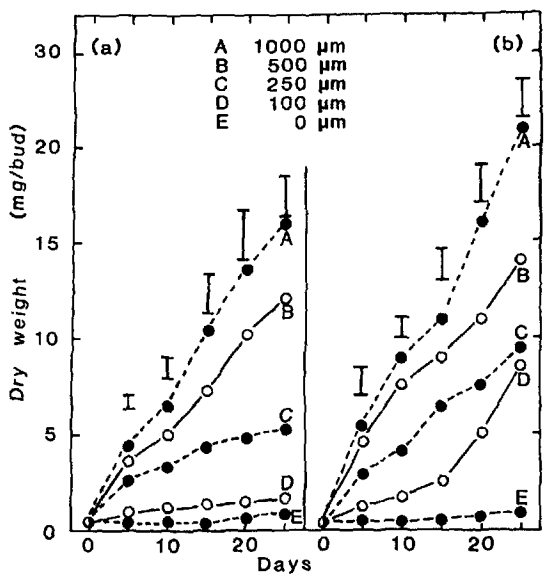


Fig. 3. Changes in dry weight of apple buds induced by (a) 1-(3,5-dichlorophenyl)-3-nitroguanidine or (b) 1-(α-ethylbenzyl)-3-nitroguanidine. Vertical bar is LSD (5%) among treatments.

The compositional changes of sterol during bud break and bud growth induced by 1-(3,5-dichlorophenyl)-3-nitroguanidine were similar to those induced by 1-(α-ethylbenzyl)-3-nitroguanidine. Therefore, the changes of sterols in the developing buds were expressed based on fresh weight increase. β-Sitosterol and sitosteryl ester were the predominant sterols (Fig. 5). Other steryl

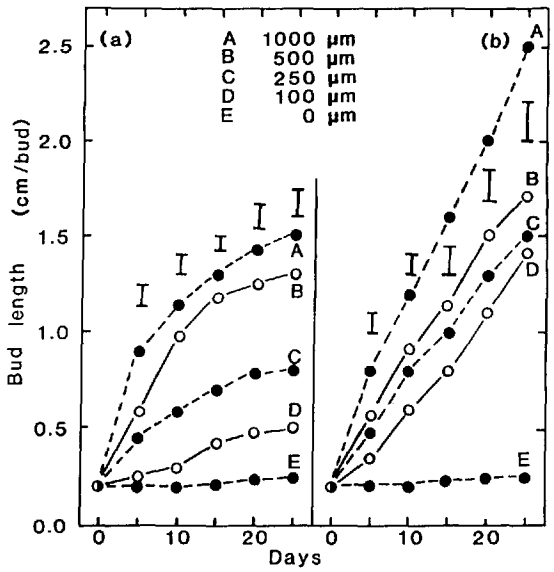


Fig. 4. Changes in length of apple buds induced by (a) 1-(3,5-dichlorophenyl)-3-nitroguanidine or (b) 1-(α-ethylbenzyl)-3-nitroguanidine. Vertical bar is LSD (5%) among treatments.

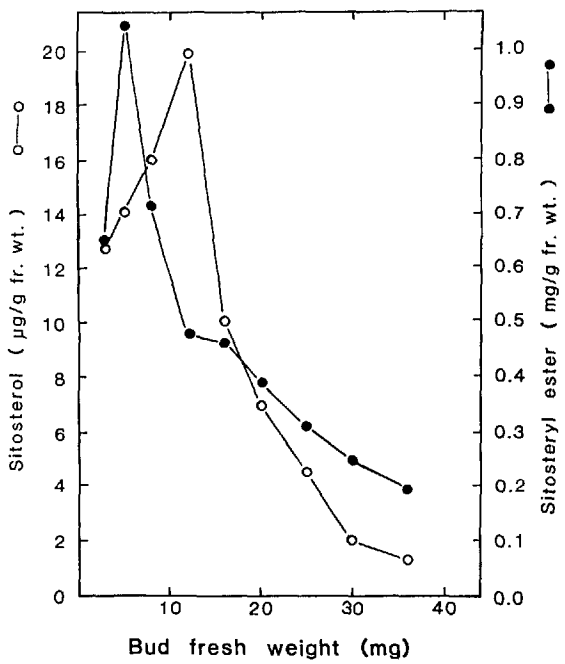


Fig. 5. Changes in sitosterol and sitosteryl ester in apple buds during bud break and resumption of growth. Data are pooled from various treatments with 1-(3,5-dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine, based on fresh weight of the buds. The first data point is for the dormant bud. LSD (5%) for sitosterol, 1.93; sitosteryl ester, 2.45.

esters were present in trace amounts (data not shown). The campesterol and stigmasterol contents were much less than β-sitosterol (Fig. 6).

The amount of sitosterol and sitosteryl ester in the buds treated with 1-(3,5-dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine in-

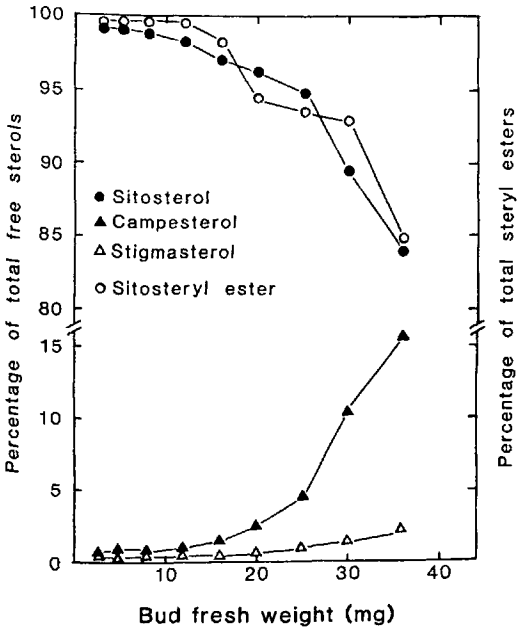


Fig. 6. Changes in percentage of individual sterols in apple buds during bud break and resumption of growth. For details see Fig. 5. LSD (5%) for sitosterol, 1.96; campesterol 2.11; stigmasterol, 1.48; sitosteryl ester, 2.17.

creased immediately after the breaking of dormancy and the beginning of active metabolism and resumption of growth and then declined (Fig. 5). A similar increase in sterol content occurred in apple buds when dormancy was broken by thidiazuron (Wang and Faust 1988). An increase in sterol content during bud break and early bud growth indicates that sterol synthesis is rapid in actively growing and differentiating plant tissue.

It has been reported that sterols appear to be associated with some physiological phenomena such as cellular differentiation (Garg and Paleg 1986, Geuns 1973, Goad 1983). Changes in sterols in the buds may be involved in the induction of other biochemical or physiological processes occurring during bud development (Wang and Faust 1988). As with free sterols, sitosteryl ester was also present in significant amounts in apple buds (Figs. 5 and 6). Steryl esters have been reported to be associated with the storage and synthesis of free sterols (Grunwald 1975). The increase in free sterols may be, in part, the result of hydrolysis of reverse steryl esters in buds. Sitosteryl ester might serve as a reserve pool for further steroidogenesis and subject to metabolic regulation to meet the changing needs of the plant cell (Wang and Faust 1988). Utilization of free sterol during rapid bud development for synthesizing cellular constituents may cause the decrease in sterol level (Fig. 5).

Decrease in relative percentage of sitosterol and sitosteryl ester was accompanied by an increase in relative percentage of campesterol and stigmasterol in buds during bud break and bud growth (Fig. 6). The changes may represent an interconversion among these three phytosterols and change in cell structure in the buds. A decrease in the ratio of free sterols to phospholipids and an increase in the ratio of campesterol + stigmasterol to sitosterol also occurred in the buds associated with resumption of growth (Fig. 7). This may indicate an

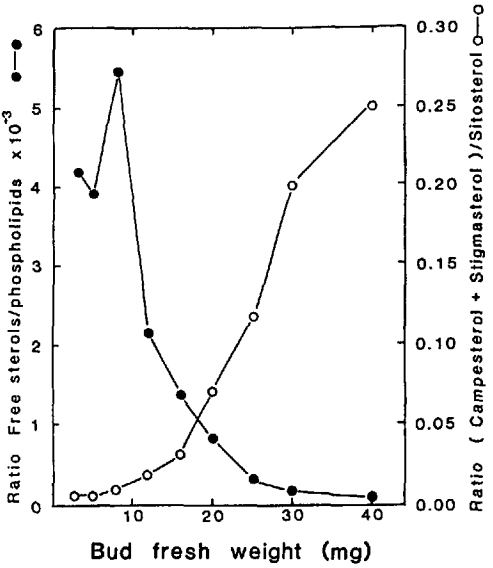


Fig. 7. Changes in the ratio of free sterols to phospholipids and campesterol + stigmasterol to sitosterol in apple buds during bud break and resumption of growth. For details see Fig. 5. LSD (5%) for ratio of free sterols to phospholipids, 0.93; campesterol + stigmasterol to sitosterol, 0.06.

increase in membrane fluidity and cell metabolism (Sikorska and Farkas 1982), a probable prerequisite for bud break and bud growth. 1-(m-Methoxybenzyl)-3-nitroguanidine had no effect on breaking apple bud dormancy and also had no effect on changes in sterol content. Whether the activation of sterol composition changes is involved in the "trigger mechanism of bud breaking" or is a consequence of dormancy-breaking mechanisms needs to be clarified by further experimentation.

Conclusion

The application of 1-(3,5-dichlorophenyl)-3-nitroguanidine or 1-(α-ethylbenzyl)-3-nitroguanidine induced bud break and a sequence of changes in the sterol composition associated with bud break and bud development. β-Sitosterol and sitosteryl ester increased immediately after bud break, then declined. A decrease in the percentage of the sitosterol and sitosteryl ester was accompanied by an increase in campesterol and stigmasterol at the beginning of rapid growth. A decrease in the ratio of free sterols to phospholipids and an increase in the ratio of campesterol + stigmasterol to sitosterol was also found after breaking of dormancy. These increases and decreases were also evident, even though to a lesser degree, when expressed on dry weight basis (data not shown). From these results it would appear that bud break may be mediated through the use of a variety of synthetic chemicals which change sterol composition and affect the overall metabolic activities of the plant.

Acknowledgments. The authors thank Ms. Amy L. Jordan for technical assistance and Dr. T. Sun of the Food Safety Inspection Service, USDA, Athens, GA, USA, for his assistance in GC-MS analysis. The authors also thank Dr. Shirley J. Rodaway of the American Cyanamid Company for

the gift of nitroguanidines. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products or vendors that may be suitable.

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