Mitotic and structural effects of nitralin and butralin on ryegrass (Lolium perenne L.) root meristems

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Received 15 March 1991
Revised version accepted 18 September 1991

Summary: Résumé: Zusammenfassung

Saturated solutions of nitralin or butralin were used to treat the germinating florets of Lolium perenne L. var. Manhattan in a growth chamber to evaluate the induced mitotic and structural effects. Examination of structural changes showed suppressed root elongation and increased radial root enlargement. The response to nitralin was observed at 1 h, whereas a weaker response was first observed with butralin at 12 h. Cortical and epidermal cells close to the apex, treated with either herbicide, were enlarged and more vacuolate. Normal mitotic figures were reduced by 76% and 36% after 1 h following treatment with nitralin and butralin, respectively. Division figures differed markedly between the two herbicides. Nitralin-treated cells formed polymorphic nuclei with an enlargement of nuclear volume and an increase in ploidy level. Butralin decreased the number of prophase division figures, but increased the number of nuclei per cell, yielding a multinucleate cell. Nitralin effects on cell divisions resemble those caused by the other dinitroaniline herbicides trifluralin, oryzalin, and pendimethalin. Butralin effects on cell division more closely resemble those reported from the carbamates, propanil and chlorpropham.

Effets mitotiques et structuraux de la nitraline et de la butraline sur les meristèmes racinaires du ray-grass (Lolium perenne L.)

Des solutions saturées de nitraline et de butraline ont été utilisées pour traiter des épillets en germination de Lolium perenne L. var. Manhattan en chambre climatique afin d'évaluer les effets mitotiques et structurels induits. L'examen des changements structurels a montré la suppression de l'elongation racinaire et l'augmentation du diamètre racinaire. La réponse à la nitraline a été observée après une heure tandis qu'une réponse plus faible a été observée avec la butraline après 12 heures. Les cellules corticales et épidémiques proches de l'apex, traitées avec l'un ou l'autre herbicide, étaient plus grandes et plus vacuolées. Les schémas mitotiques normaux ont été réduits de 76% et 36% une heure après un traitement à la nitraline et à la butraline, respectivement. Les schémas de division différaient de façon marquée entre les 2 herbicides. Les cellules traitées à la nitraline ont formé des cellules avec des nucléï polymorphes avec augmentation du volume nucléaire et du niveau de ploïdie. La butraline a limité le nombre de stades prophasiques mais a augmenté le nombre de nucléï par cellule, aboutissant à une cellule multinucléée. Les effets de la nitraline sur la division cellulaire ressemblent à ceux causés par les autres herbicides dinitroanilines: trifluraline, oryzaline, et pendiméthaline, et les effets de la butraline sur la division cellulaire sont plus voisins de ceux rapportés pour les carbamates, propanil et chlorpropham.

Wirkungen von Nitralin und Butralin auf Mitose und Struktur des Wurzelmeristems von Deutschem Weidelgras (Lolium perenne L.)

Nach der Behandlung keimender Samen von Lolium perenne L. 'Manhattan' mit gesättigten Lösungen von Nitralin und Butralin im Phyto- tron entwickelten sich verkürzte, aber verdicke Wurzeln. Bei Nitralin wurden nach 1 h Wirkungen beobachtet, bei Butralin in geringerem

**Introduction**

The compounds nitralin and butralin are members of the dinitroaniline family of herbicides, which also includes trifluralin, oryzalin and pendimethalin. The dinitroaniline herbicides are valuable tools for the control of grasses and certain broadleaf weeds in cotton (*Gossypium hirsutum* L.), soybeans (*Glycine max* L.), and tree, vine and ornamental crops. They have been reported to have a similar mode of action in plants because they are non-mobile, inhibit root growth, and are mitotic poisons (Harvey, 1973; Beuret, 1980; Malefy & Duke, 1982; Upadhyaya & Nooden, 1987). In an illustrative study, Upadhyaya & Nooden (1987) analysed the effects of several dinitroaniline herbicides, including trifluralin and oryzalin, on seedlings of corn (*Zea mays* L., 'Michigan 200 hybrid'). When applied at concentrations of $10^{-4}$ M, the herbicides inhibited cell elongation and promoted isodiametric cell enlargement in the elongation zone of root tips. Root length was reduced by 50%, compared to the control, and root tips became thickened and club-shaped. Mitosis in the meristem and zone of elongation was disrupted, resulting in many cells containing abnormally enlarged or multiple nuclei.

As with other dinitroaniline herbicides, nitralin at low concentrations has a pronounced inhibitory effect on the development of roots and shoots. Barrentine & Warren (1971) reported that roots of many broadleaf and grass species, including the crop plants soybean (*Glycine max* 'Amsoy'), wheat (*Triticum aestivum* L. 'Dual Miller'), oats (*Avena sativa* L. 'Jaycee'), barley (*Hordeum vulgare* L. 'Harrison') and sorghum (*Sorghum bicolor* (L.) Moench 'RS-6(0)'), and the roots of barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) and ivyleaf morning glory (*Ipomoea hederacea* (L.) Jacq.), were more sensitive than the shoots. In these cases, the concentration of nitralin necessary to reduce the length or fresh weight of shoots by 50% ranged from 3 to 300 times the concentration necessary for roots.

Grass plants are more sensitive to nitralin treatment than broadleaf plants. In one study, cultured wheat roots were inhibited by herbicide concentrations of 0.02 ppm (Hess & Bayer, 1974), whereas Lund *et al.* (1970) found that concentrations of 0.5-1.0 ppm were required to reduce the yield of cotton root tissue by 50%. Similarly, Barrentine & Warren (1971) reported that while 0.08 ppm nitralin halved the root growth of wheat and oat seedlings grown in sand culture, the herbicide needed to be applied at 0.74 ppm to have the same effect on soybean roots.

Butralin stunted root growth of soybean (*Glycine max* (L.) Merr 'Corsoy') and velvetleaf (*Abutilon theophrasti* Medic.) less severely than trifluralin, nitralin or oryzalin when evaluated at equal concentrations *in vitro* (Harvey, 1973). Bishop *et al.* (1971) reported that butralin was relatively non-injurious to cotton, peanut (*Arachis hypogea* L.) and soybeans. When applied to foxtail millet (*Setaria italica* L. Beauv.) *in vitro*, butralin reduced root length similarly to nitralin, although a higher effective treatment concentration was required.

Gentner & Burk (1968) reported the formation of 'digitate to globose' swellings at the tips of nitralin-inhibited corn (*Zea mays* 'U.S. 13') roots. The cells in these roots contained abnormal nuclear configurations: some possessed compacted, polyploid chromosomes, while others contained 2-5 nuclei. Normand *et al.* (1968) and Rizk (1972) both found that
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In nitralin-treated cultured cotton roots the number of mitotic figures was reduced by 95%.

Using cultured *Tradescantia paludosa* stamen hairs, Sawamura & Jackson (1968) observed mitotic aberrations when cells were treated with 0-1-5-0 ppm nitralin during early prophase. Chromosomes became indistinct and mitosis was prolonged. Cells possessing nuclei with doubled chromosome complements, and multinucleate cells with multisepta were common. Cell-plate formation was inhibited at concentrations of 3 ppm, resulting in polymorphic cells. In roots, such cells would be unable to sustain normal development, and consequently stunting would develop.

To a lesser extent, nitralin also disrupted mitosis and cytokinesis in broadbean (*Vicia faba* L.) leaf cells. Early prophase cells treated with 0-3-3-0 ppm nitralin regressed to interphase. Cells in later mitotic phases, when treated, were able to complete nuclear division, but unable to effect cytoplasmic partitioning. Cells treated in anaphase formed a phragmoplast and cell plate which eventually disintegrated, producing a binucleate cell. Nitralin also prevented cells in telophase from completing cell plate formation. Binucleate cells thus created by herbicide treatment would be unable to divide further and develop in the organized manner necessary for growth and development.

The aim of this study is to describe in detail the anatomical and mitotic changes produced in ryegrass root meristems over a 96-h period of treatment with nitralin or butralin.

**Materials and methods**

**Culture conditions**

Florets of *Lolium perenne* L. Manhattan, solely composed of diploid seeds, were germinated on 200 ml of 1/16th strength Hoagland's nutrient solution (Hoagland & Amon, 1950), adjusted to pH 7-2, in Pyrex dishes. The florets were maintained in a growth chamber under a regime of 16-h, 23°C days, and 8-h, 20°C nights, with a light intensity of 200 µmol photons m⁻² s⁻¹. The solutions were changed daily.

**Herbicide preparation**

Excess technical-grade nitralin (97% pure) was added to 1/16th strength Hoagland's solution (pH 7-2) in an aluminum foil-wrapped flask. The mixture was stirred constantly while being heated in a water bath to 75°C, and was then cooled slowly in a second water bath for approximately 2 h. The solution, representing 0-6 ppm at 25°C, was shaken vigorously overnight before use. Stock solutions were stored in the dark between experiments for up to 96 h.

Excess technical-grade butralin (98% pure) was added to a 1/16th strength Hoagland's solution (pH 7-2) in an aluminum foil-wrapped flask. The resulting mixture was stirred constantly while being heated in a water bath to 50°C, and was then cooled slowly in a second water bath for approximately 2 h. The solution, representing 1 ppm at 25°C, was shaken vigorously overnight before use. Between experiments, stock solutions were stored in the dark for up to 96 h.

**Treatment**

Radicles emerged from the seeds to a length of about 2 mm after 72 h of culture. Herbicide treatments were then initiated by replacing the nutrient solution with the herbicide solution. Samples of tissue for anatomical and histochemical studies were taken at intervals of 1, 4, 12, 24, 48 and 96 h.

**Preparation of tissue for light microscopy**

The apical 2 mm of each seedling root were excised, fixed for 24 h in a solution of formalin, acetic acid and ethanol (1:1:18, v/v/v) (Johansen, 1940), dehydrated in a tertiary butyl alcohol series (Jensen, 1962), and embedded in Paraplast. Sections were cut at 9 or 10 µm.

Alternate apices were fixed for 2 h in a 4% solution of 25% commercial grade glutaraldehyde in 0.1 M Na-K phosphate buffer (pH 7-2). This was followed by dehydration in a graded acetone series. The apices were then embedded in a modified Spurr's resin (Spurr, 1969; Lin et al., 1974).

**DNA histochemistry**

Sections were hydrolysed at 60°C for 12 min, and then immersed in Schiff's reagent for 5 h in the dark. Treatment of tissue with Schiff's reagent after hydrolysis of cellular RNA with HCl
is recognized as being specific for the identification of exposed aldehyde groups of DNA (Pearse, 1968). After staining, the sections were passed through three 10-min changes of a solution of 5 ml of 10% K₂S₂O₅ and 5 ml of 1N HCl in 100 ml of distilled water.

_Cell/nuclear volume (cv/nv) ratio_

A minimum of three root samples were taken for each treatment interval. Cells from the first and second cortical layers at a distance of 0.25 mm from the root cap were measured. The average of two diameters at right angles to each other of 10 cells and their nuclei was taken. When calculating the nuclear volume, the nucleus was assumed to be spherical.

When measuring micronuclei in mother cells treated with butralin, each micronucleus was measured and the average total volume calculated for the entire mother cell. DNA content per cell was estimated from the nuclear volume measurements using the relationship log DNA/N (nucleus) = 1.497 + 1.22 log nv (Baetcke et al., 1967). An estimate of the ploidy number was made from the data. The percentage of multinucleate cells was determined by counting the number of nuclei per cell. Ten cortical cells from each of the four roots were counted.

_Mitotic stages_

Mitotic stages in 9-µm sections from a minimum of 13 samples were counted. The number of cells in each mitotic stage and interphase was determined as a percentage of the total number of cells counted. Abnormal, clumped chromosomes in nitralin-treated cells were counted separately from normal metaphase figures. Normal and abnormal mitotic figures were counted and expressed as a percentage of the number of normal cells in mitosis.

_Results and discussion_

_Control roots_

Germination and growth of control florets was rapid, yielding radicles approximately 2 mm long after 72 h. After 96 h of treatment, control roots had elongated to 13.8 mm (Fig. 1a). The root diameter at the zone of elongation was 0.25 mm throughout this period (Fig. 1b).

The root apical meristem was composed of three groups of initials. The outermost group of initials gave rise to the root cap which surrounded the meristem. The root cap was composed of parenchyma cells that possessed small, uniform nuclei, and contained a large number of amyloplasts (Fig. 3).

The root hair development in the trichoblasts was initiated approximately 0.32 mm from the apex; the root hairs themselves became visible to the naked eye at a distance of 0.9 mm from the root cap (Fig. 1c).

Four to six layers of isodiametric cortical cells with dense cytoplasm and large nuclei populated the cell layers directly beneath the epidermis. As elongation proceeded, the cortical cells became more vacuolate. The innermost cortical layer formed the endodermis, the layer of cells surrounding the vascular cylinder.
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The third, innermost set of initials formed the vascular cylinder. Within the latter, differentiation of sieve elements could be observed to commence 0.18 mm from the apex. Differentiated protophloem sieve elements were apparent 0.23 mm from the apex, in the region where elongation was still pre-eminent.

The untreated roots exhibited a normal course of growth and development in the types of tissue produced, the cytological characteristics of those tissues, and the distance over which the tissues differentiated from the meristematic cells of the apex.

Treated roots

Both herbicides suppressed root elongation growth throughout the treatment period (Fig. 1a). After 96 h, roots treated with nitralin were 72% shorter than controls; butralin-treated roots were 67% shorter.

The decrease in length was accompanied by radial enlargement of the roots in a manner that resembled the 'digitate' corn roots observed by Gentner & Burk (1968) after nitralin treatment. Conclusions regarding which region of the root exhibits this enlargement have differed among researchers. In the present study, enlargement (Fig. 1b) occurred principally in the zone of differentiation. This site of effect was the same as that observed by Bayer et al. (1967) in trifluralin-treated cotton roots. However, Lignowski & Scott (1971) reported that wheat roots treated with trifluralin exhibited the greatest radial expansion in the region of elongation. Upadhyaya & Nooden (1987) also found that the radial enlargement produced by several dinitroanilines, including trifluralin and oryzalin, was centred in the elongating tissues of corn roots.
Enlargement was observed after 1 h of nitralin treatment, and culminated in a 219% increase in root diameter after 96 h. The effects of butralin were slower to develop and smaller in magnitude. Enlargement was observed to occur 12 h after butralin treatment, and after 96 h the root diameter exhibited a 119% increase.

Cellular effects

When sections of the treated tissues were observed with the light microscope, the cellular basis of the visible radial enlargement became evident. Cortical cells in the nitralin-treated meristems increased in volume 37·4-fold over a period of 48 h. Their nuclear volume increased at a slower rate, enlarging 13·4-fold over the same time period. Similar cells in butralin-treated meristems exhibited a 17·6-fold cellular enlargement and a 11·1-fold nuclear enlargement over a period of 48 h (Figs 4b, 4c). The ratio of cell volume to nuclear volume almost doubled after nitralin treatment, but butralin treatment caused only a minor change (Table 1). This radial or isodiametric expansion of root cells proximal to the root apical meristem is an effect of dinitroaniline herbicides that has often been reported. It was observed in trifluralin-treated corn and cotton roots by Amato et al. (1965) and Lignowski & Scott (1971). Dowidar & El-Nahas (1978) found that epidermal, cortical and pith cells in the elongation zone of onion root tips increased in volume by 2·5- to 4·5-fold after trifluralin treatment. Malefyt & Duke (1982) also reported that pendimethalin, applied to velvetleaf (Abutilon theophrasti Medic.) and pigweed (Amaranthus sp.), caused radial and longitudinal expansion of cortical, epidermal and pericycle cells located 300-1500 µm from the root tip.

Herbicide treatment also affected cellular differentiation. The division of meristematic initials was inhibited, but cells proximal to the apex continued to differentiate and mature. As indicated by Ashton & Crafts (1973) and Hess (1983), increased differentiation is often a characteristic of inhibited roots in which mitosis has been disrupted. As the treatment continued, differentiated cells increased, and developmental stages appeared closer than usual to the apex. Cortical and epidermal cells in root tips treated with either nitralin or butralin were enlarged and more vacuolated closer to the apex than cells in untreated roots. Xylem development or, more specifically, the elongation of nuclei in developing xylem elements, also appeared earlier. Normand et al. (1968) obtained similar results when they treated cultured cotton roots with nitralin: more xylem differentiation was observed in cross-sections after treatment.

Root hair growth was not inhibited, but differentiation of trichoblasts increased exponentially with increasing duration of treatment (Fig. 1c). Root hairs, which in untreated roots were initiated at a distance of 0·90 mm from the apex, developed within 0·26 mm of the apex in nitralin-treated roots and within 0·29 mm of the apex in butralin-treated roots. The hairs elongated beyond the root cap (Fig. 4c).

Root-cap cells in herbicide-treated meristems showed no abnormal enlargement, although the number of such cells decreased. The number of amyloplasts per cell also decreased.

Cell division effects

Nitralin and butralin each affected cell division in treated roots in a different manner. Normal mitotic figures were reduced by 76% after 1 h of

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**Table 1. Effect of nitralin and butralin on cell volume to nuclear volume (cv/nv) ratio, nuclear volume, DNA/nucleus and estimated polyploid number**

<table>
<thead>
<tr>
<th>Duration of treatment (h)</th>
<th>Volume ratio (CV/NV)</th>
<th>Nuclear volume (µm³)</th>
<th>DNA/nucleus (µg)*</th>
<th>Polyploid number</th>
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<td>Butralin</td>
<td>Nitralin</td>
</tr>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>110·0</td>
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<td>4·38</td>
<td>3·01</td>
<td>101·1</td>
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<td>4·06</td>
<td>5·93</td>
<td>5·25</td>
<td>120·0</td>
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<tr>
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<td>7·79</td>
<td>4·14</td>
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<td>5·12</td>
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<tr>
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<td>3·18</td>
<td>8·61</td>
<td>4·25</td>
<td>1897·5</td>
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</table>

*Log DNA/nucleus (in pg) = 1·497 + 1·22 log NV.
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Fig. 4. Effect of nitralin and butralin on cortical cells of the root apex of ryegrass: (A) condensed chromosomes (arrows) 1 h after nitralin treatment; (B) enlarged cells and polymorphic nuclei (arrows) after nitralin treatment; (C) butralin-treated apex with enlarged epidermal and cortical cells. Note sections of root hairs by the root cap (bars = 10 µm).

nitralin treatment (Figs 2a, b, and 4a); by 12 h of treatment, normal division figures were no longer present. Abnormal mitotic figures began to appear 1 h after treatment (Fig. 4a), at which time they represented 3.3% of the total number of cells in mitosis. After 12 h, cells with aberrant mitotic figures represented 7.9% of the population. Aberrations included 'C' mitotic figures similar to those produced by colchicine treatment and arrested metaphase figures. Dinitroaniline herbicides have previously been observed to produce mitotic abnormalities. Trifluralin induced 'C' mitotic figures in wheat and onion (Allium cepa 'Yellow Globe') root
meristems (Lignowski & Scott, 1972). Malefyt & Duke (1982) reported that pendimethalin treatment appeared to 'freeze' prophase chromosomes of velvetleaf and pigweed root tip cells into tightly condensed balls. However, nitralin produced effects more rapidly (within 1 h) than the other two herbicides: trifluralin was reported to exert effects after 4 h (Hess & Bayer, 1974), and pendimethalin-treated cells exhibited abnormalities within 4-8 h after the commencement of treatment (Malefyt & Duke, 1982). These differences may be species-specific rather than herbicide-specific.

Nitralin disrupted chromosome movement in cells in the early stages of mitosis and inhibited cell-plate formation in cells in telophase. As a result, treated cells entering mitosis were unable to complete the process. Although Gentner & Burk (1968) reported some division through anaphase in nitralin-treated corn roots, ryegrass cells were inhibited before anaphase commenced. Thus, as the treatment continued, the number of cells in prophase increased at a greater rate than the number of cells in telophase (Fig. 2b). After 12 h, cells in prophase represented 13.3% of the population. Cells in telophase were absent. The small number of cells that had been in telophase when treatment began underwent incomplete cell-plate formation and formed a polymorphic nucleus (Fig. 5b). Talbert (1965), using trifluralin on cultured soybean roots, found that prophase figures also increased relative to the control plants.

Although cells were prevented from completing mitosis and cytokinesis, DNA replication continued, leading to endopolyploidy. Nuclear volume measurements indicated an increase in

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Fig. 5. Effect of nitralin and butralin on cells of the root apex of ryegrass: (A) multinucleate cells with segments of cell wall after 24 h of butralin treatment; (B) polymorphic nuclei (N) after 48 h of nitralin treatment (bars = 2 µm).

Fig. 6. Effect of nitralin and butralin on cortical cells of root apex of ryegrass: (A) tightly condensed chromosomes, 'ball nuclei' (arrow), formed after 48 h of treatment with nitralin (bar = 4 µm); (B) cortical cells with multipolar spindle (arrow) after 48 h of butralin treatment (bar = 2 µm).
the ploidy level to 64 N after 48 h of treatment (Table 1). Many polyploid cells exhibited ball metaphase figures (Fig. 5a). The aggregation and continued duplication of chromosomes supported Sawamura & Jackson's (1968) hypothesis that nitralin causes mitotic aberrations similar to those produced by colchicine. Other nuclei in the meristem were polymorphic (Fig. 5a), but no polynucleate cells were observed in thin sectioned material. This finding is in contrast to the results reported for nitralin-treated stamen hairs (Sawamura & Jackson, 1968) and trifluralin (Talbert, 1965; Hacskaylo & Amato, 1968; Lignowski & Scott, 1972; Hess & Bayer, 1974). The limits of resolution of the light microscope may account for the discrepancy between the results.

Butralin decreased the percentage of normal mitotic figures by 35% after 1 h of treatment, by 60% after 4 h of treatment and by 90% after 24 h of treatment. However, normal division figures were infrequently observed even after 48 h of treatment.

Butralin treatment decreased the number of prophase division figures, but increased the number of nuclei per cell (Figs 2c and 5a). In dividing cells, paired chromosomes separated and the nuclear envelopes reformed. However, apolar division without cytokinesis occurred (Figs 5a and 6b). The chromosomes became randomly distributed in the cell. After 24 h of treatment, these cells became multinucleate with several nuclei. The micronuclei were divided wholly or partially by fine, randomly located cell walls within the mother cell wall (Fig. 5a). Ploidy levels within the mother cells increased to 40 N after 48 h (Table 1).

The disruption of mitosis by nitralin resembles the results obtained with trifluralin (Amato et al., 1965; Hess & Bayer, 1974; Hess, 1983), oryzalin (Barrels & Hilton, 1973) and colchicine (Derman, 1938). The mitotic aberrations caused by butralin more closely paralleled those produced by propanol (isopropyl carbamate) and chlorpropham (isopropyl m-chlorocarbanilate) (Ennis, 1948; Calvin & Friesen, 1959; Sawamura, 1965; Hess, 1983). Apolar divisions or a multipolar spindle apparatus have been described in endosperm cells treated with propanol by Hepler & Jackson (1969). Abnormal cell wall formation similar to that caused by butralin was observed by Holmsen & Hess (1984) in oat (Avena sativa L.) roots treated with 5-6 M DCPA.

References


