# Polyphenol and Alkaloid Changes in Glyphosate-Treated Tobacco Regenerants Selected for Herbicide Tolerance

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*In vitro* a one-step selection for glyphosate tolerance of two Bulgarian tobacco cultivars Zlatna Arda and Nevrokop A24 was carried out. After treatment with glyphosate of the control plants and their regenerants polyphenol and alkaloid changes were determined. A decrease in the polyphenol concentrations and an increase in the alkaloid concentrations were registered only for Zlatna Arda.

## Introduction

A large number of data exist concerning the action of glyphosate on plants and tolerant forms obtained via various approaches (Della-Chiopa *et al.*, 1987; Fillatti *et al.*, 1987; Hauptmann *et al.*, 1988; Jordan and McHoughen, 1988). The herbicide inhibits protein and nucleic acid synthesis, photosynthesis, transpiration and polyphenol biosynthesis (Cole, 1985).

Polyphenols and alkaloids are often connected with plant defence against predators, bacteria and fungi. In this paper we discuss the changes in the concentrations of polyphenols and alkaloids in two Bulgarian tobacco cultivars and their regenerants after the action of glyphosate. Zlatna Arda was chosen because of the excellent processing quality and Nevrokop A24 because of its complex disease resistance (Anon., 1990). One-step and step-wise in vitro selection procedures with glyphosate were carried out. Regenerants were obtained only after the one-step selection.

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## Results and Discussion

### *Plant experiments*

The callus growth index (GI) (ratio of final to initial fresh weights of the cultures), was used to express the influence of glyphosate. Significant differences were observed between values of the GI when the growth of the untreated calli (control) was compared to those of calli grown on various glyphosate concentrations in the medium. The callus growth was strongly (3 to 6 times) inhibited in the presence of glyphosate. The calli maintained for 30–45 days at lower glyphosate doses (5–40 mM) still included green and friable islands, while at higher doses (60–80 mM) they turned brownish and leaky.

Plant regeneration was strongly inhibited during the one-step selection procedure. We succeeded to regenerate only few plants that were cloned and potted. Regenerants (one per genotype) were used for the further biochemical analyses.

At the step-wise selection the growth of the Zlatna Arda cultures was strongly suppressed even at 5 mM glyphosate in the regeneration medium (Table I.). Comparable GI values for the Nevrokop A24 cultures were reached only at 60 mM glyphosate. The regeneration was fully in-

Table I. Growth index (GI) of the culture maintained on increasing concentrations of glyphosate in the callus medium at the step-wise selection procedure.

| Glyphosate [mM] | GI*, Nevrokop A24 | | GI, Zlatna Arda | |
|---|---|---|---|---|
| | Control | Herbicide | Control | Herbicide |
| 0 | 13.9 ± 0.5 | – | 15.2 ± 0.4 | – |
| 5 | 11.2 ± 0.5 | 3.1 ± 0.2 | 3.1 ± 0.4 | 2.0 ± 0.3 |
| 10 | 7.0 ± 0.2 | 2.1 ± 0.2 | 2.7 ± 0.1 | 1.5 ± 0.3 |
| 20 | 3.1 ± 0.2 | 2.0 ± 0.2 | 2.0 ± 0.2 | 1.0 ± 0.1 |
| 40 | 3.3 ± 0.1 | 1.8 ± 0.1 | 1.5 ± 0.2 | 1.0 ± 0.1 |
| 60 | 2.1 ± 0.1 | 1.5 ± 0.1 | 1.2 ± 0.1 | 0.9 ± 0.1 |
| 80 | 1.5 ± 0.2 | 1.2 ± 0.1 | 1.0 ± 0.1 | 0.9 ± 0.1 |

* GI- ratio between the final and the initial fresh weights of the cultures.

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hibited even at the lowest herbicide concentration in the medium for both of the genotypes.

The data from the *in vitro* experiments showed that in the system used the continuing presence of glyphosate in the media regardless of the concentration fully inhibited the regeneration. When the herbicide was added only to the callus medium, it was possible to select regenerants that tolerate field doses.

*Polyphenol and alkaloid changes after glyphosate treatment*

Significant differences were observed in the concentrations of some secondary metabolites. The main polyphenols in Zlatna Arda appeared to be the flavonoid glycoside rutin and chlorogenic acid. In Nevrokop A24 only chlorogenic acid was detected (Table II). The alkaloid fraction of both cultivars consisted mainly of nicotine (above 90%). The concentration of the alkaloids was higher in Nevrokop A24 (Table II).

The treatment with glyphosate radically influenced the secondary metabolism only of the cultivar Zlatna Arda. In these plants the concentrations of polyphenols decreased while those of the alkaloids increased (Table I). A sharp increase in the alkaloids in the regenerants was determined. The decrease in the concentration of one defensive compound (chlorogenic acid) was accompanied by the increase in the concentration of another defensive compound (nicotine). Probably such changes could proceed in order to keep up the plant resistance.

Table II. Polyphenols and alkaloids in the investigated tobacco plants.

| Plant | Chlorogenic acid [%]* | Rutin [%] | Alkaloids [%] |
|---|---|---|---|
| Zlatna Arda | | | |
| Control | 0.62 ± 0.03 | 0.27 ± 0.03 | 0.09 ± 0.01 |
| Treated cultivar | 0.13 ± 0.03 | trace | 0.50 ± 0.01 |
| Regenerant | 0.45 ± 0.02 | 0.00 | 1.46 ± 0.07 |
| Treated regenerant | 0.29 ± 0.03 | 0.00 | 1.20 ± 0.05 |
| Nevrokop A24 | | | |
| Control | 0.47 ± 0.06 | 0.00 | 0.23 ± 0.01 |
| Treated cultivar | 0.34 ± 0.04 | 0.00 | 0.19 ± 0.01 |
| Regenerant | 0.32 ± 0.02 | 0.00 | 0.25 ± 0.02 |
| Treated regenerant | 0.28 ± 0.04 | 0.00 | 0.55 ± 0.02 |

* Percentage of each component ± standard deviation (triplicate determinations).

Negligible changes occurred in the cultivar Nevrokop A24. Only the alkaloid concentration in the herbicide treated regenerants increased, but in a less dramatic way than in Zlatna Arda.

The chemical data are in agreement with the results obtained in the plant experiments, showing that Nevrokop A24 is to some extent more tolerant to the herbicide.

## Materials and Methods

*Plant material*

Two oriental type Bulgarian tobacco cultivars Zlatna Arda and Nevrokop A24 were used for callus induction of aseptically maintained seedlings.

*In vitro procedure*

Micropropagation and maintaining by node cuttings on hormone-free MS medium (Murashige and Skoog, 1962); callus induction of stem cuttings on MS medium supplemented with casein hydrolyzate (Sigma) (500 $mg \cdot l^{-1}$), naphtaleneacetic acid (Serva) (1 $mg \cdot l^{-1}$) and kinetine (Serva) (0.5 $mg \cdot l^{-1}$); regeneration (I stage) on MS with casein hydrolyzate (250 $mg \cdot l^{-1}$), indole-3-acetic acid (Sigma) (0.2 $mg \cdot l^{-1}$), kinetine (0.5 $mg \cdot l^{-1}$) and II stage – on MS with casein hydrolyzate (500 $mg \cdot l^{-1}$), IAA (0.2 $mg \cdot l^{-1}$), kinetine (1 $mg \cdot l^{-1}$) and gibberelic acid (Sigma) ($mg \cdot l^{-1}$).

*Selection procedure for herbicide tolerance*

*One-step* – addition of glyphosate only to the callus medium where the cultures were maintained for 4 - 5 weeks. Regeneration from these calli was performed on herbicide-free medium.

*Step-wise* – glyphosate presence in both callus and regeneration media. From the herbicide-containing callus medium the cultures were transferred for another 4–5 weeks to regeneration medium.

The herbicide was filter sterilised and added to the media in 5, 10, 20 or 80 mM doses.

The *in vitro* experiments were repeated three times. Every repetition included at least 10 tubes (samples) per glyphosate concentration

The regenerants, obtained only after the one-step selection procedure at 20 mM glyphosate, were cloned and planted into soil under greenhouse conditions.

Micropropagated seedlings from both cultivars were used as controls during the whole procedure. When the potted plants (regenerants and controls) reached a 7–8 leaf stage, half of them (10–15 per genotype) were sprayed with 20 mM (field dose) of glyphosate solution. Four days after the treatment all leaves of the sprayed and unsprayed plants were collected for analysis of polyphenols and alkaloids.

*Analysis of polyphenols*

Chlorogenic acid and rutin were determined simultaneously using a spectrophotometric method based on the bathochromic shift of the adsorption spectrum induced by aminoethyl diphenylborate (Hausermann and Waltz, 1962).

*Analysis of alkaloids*

To 1 g dried tobacco leaves 40 ml of saturated NaCl solution were added, 8 ml 8 N NaOH and 5–25 ml distilled water. After steam distillation 250 ml distillate was collected. An aliquot (25 ml) was diluted to 100 ml with 0.05 N $H_2SO_4$ and spectrophotometrically analysed at $\lambda_{min}$ 236, $\lambda_{max}$ 259 and $\lambda_{min}$ 282 nm.

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