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**Original Research Article**

# Anatomical Changes in Tobacco Leaf after Treatment with Isoxaflutole

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**A b s t r a c t**

The toxicity of the Isoxaflutole on the anatomical structure of the leaf in tobacco (*Nicotiana tabacum* L.) was studied by light microscopy. In the areas treated during vegetation, the plants showed visible morphological signs of phytotoxicity consisting of chlorosis, deformation of the leaf lamina and inhibition of growth. Histological these changes consist in a reduction of the number/mm² of stomata on both upper and lower epidermal surfaces, deformation of the guard cells of stomata and reduction of the total thickness of assimilation parenchyma (µm) - mesophyll.

**K e y w o r d s**

Isoxaflutole
Leaf anatomy
Mesophyll
*Nicotiana tabacum*
Stomata

## Introduction

Tobacco (*Nicotiana tabacum* L.) is an important industrial crop of family *Solanaceae*. Its main purpose is determined by the content of the alkaloid nicotine in the leaves which exerts a certain impact on the human body. This specificity in its chemical composition defines it as a crop that has been grown for generations in many parts of Bulgaria. The yield, quality, and profitability of the raw material obtained from *Nicotiana tabacum* L. largely depends on proper crop rotations, agricultural techniques and timely weed control (Atanasov and Nesterov, 1981). As is known, weeds lead to reduced growth of the species and it is a host to many diseases and pests (Bukan et al., 2011); it is being extremely important to keep the plants without weeds. In this regard, the controlled use of herbicides is critical for obtaining high yields. Kalinova (2010) found that the use of the five most commonly used herbicides for tobacco results in a 27% increase in yield, and a 6.5% increase in the percentage of 1st class, on average. When the conditions for the growth and development of tobacco are more favorable, the application of herbicides has less effect on the yield.

Very often, improper use of chemical agents in agriculture, including herbicides, causes irreversible consequences and death of the plants as they become a part of their general biochemistry (Kamble, 2007). As a result of the change in the metabolism of the plant, series of physiological, anatomical and morphological changes occur which are responses of the species to the changed conditions (Corre, 1983a; 1983b; Shoch et al., 1984).

Different metabolic changes resulting from the use of different herbicides for tobacco are reflected in the work of a number of authors (Lee et al., 1998; Camper et al., 2003; Darwish et al., 2013). The above authors found decreased photosynthetic processes, inhibition of the

synthesis of chlorophyll and carotenoids, reduced peroxidation and pigmentation, resulting in inhibited growth of the plants. Mostowska (1999), in a study of the effect of herbicides on the protoplast of cells, found aging of chloroplasts coupled with damage of their membranes and intensive accumulation of plastoglobules. The same author underscores that these structural changes in protoplasmic organelles are responsible for the inhibition of photosynthesis and photodestruction of pigments. Yang et al. (2006) and Jung et al. (2008) obtained similar results while studying the influence of Oxyfluorfen. They point out that the reduction of chloroplasts and the parenchyma in leaves is the reason for the lag in the growth and development of treated plants.

In the study of the effect of some soil herbicides - Goal E2, Harness and Pulsar 40, Anastasov and Kalinova (2009a, b), Anastasov (2010a, b, c), recorded unidirectional changes in the anatomical structure of tobacco leaves (*Nicotiana tabacum* L.). They consist in a reduction in the number of stomata; atrophy of their guard cells, as well as in a reduction of the total thickness of the assimilation parenchyma.

Merlin Flex 480 SC (Isoxaflutole) is a soil herbicide which has not been registered in Bulgaria to combat tobacco weeds (*Nicotiana tabacum* L.). When introducing new herbicides, it is necessary to ascertain what their effectiveness and selectivity with respect to the main crop. The purpose of this study is to determine the biological effectiveness and selectivity for tobacco of the Isoxaflutole which is the active substance in soil herbicide, Merlin Flex SC 480.

## Materials and methods

During the period 2012-2013, in the experimental field of the Tobacco Institute – Markovo, in humus-carbonate soil, a field experiment was carried out to establish the biological effectiveness and selectivity of the Isoxaflutole, as soil systemic herbicide Merlin Flex 480 SC. The herbicide Merlin Flex480 SC contains Isoxaflutole + Cyprosulfamide (240 + 240 g/l). The Isoxaflutole (42 ml/dka) was used on 21.05.2012. The tobacco plants cv. Plovdiv 7, were transplanted on 26.05.2012. The materials for the anatomical analysis were collected on 12.06.2012 and were studied following the standard methods of comparative anatomy (Metcalfe and Chalk, 1979; Nikolov and Daskalov, 1966). The leaf samples were taken in the vegetative period of the vegetation phase, determined according to the methodology of Beydeman (1954), $6^{th}$-$7^{th}$ leaf. Samples were taken from 50 damaged plants and from the untreated (control) plants. Plants material was fixed, in accordance with Hodgson et al. (1993), in FAA for 24 h (formaldehyde, glacial acetic acid, ethyl alcohol and distilled water in a ratio of 6:1:20:40). After 24 h, the samples were washed with 50% ethyl alcohol, in three series, and then placed for storage in FA (70% ethyl alcohol and 40% formaldehyde in a ratio of 19:1).

The epidermis and transverse section were studied from the middle part of the lamina of untreated leaves (control) and leaves damaged by the herbicide. Semi-stable microscopic preparations were made. For the light microscopic examination an "Amplival" was used. Measurements were made with an eyepiece micrometer (10x) and the pictures were taken with a light digital microscope Motic DMBA-210.

The number and location of stomata are listed per mm² on the upper (ad) and lower (ab) epidermis, as well as the width and length of the guard cells of the stomata in μm at a magnification of 16x40 (eyepiece x lens). At the transverse section a total thickness of the mesophyll was measured in μm at a magnification of 16x10 (eyepiece x lens). The data were processed mathematically according to the descriptive statistics method, the quantitative indicators including: arithmetic mean (average), standard error (st. err.), standard deviation from the mean (st. dev.), coefficient of variation (Sg%), max:min index. Programs were used for data processing Statistica for Windows (Statsofting, 2007). For each indication 50 measurements were made.

## Results and discussion

During the conduct of the experiment, visible signs of phytotoxicity were observed in tobacco plants (*Nicotiana tabacum* L.). Its morphological manifestations are expressed in an inhibited growth of the plants, deformation of the leaf lamina and change in its pigmentation, chlorosis and necrotic spots (Fig. 1).

From the anatomical examination conducted, it was found that the epidermis of the leaf is formed by one row of cells, both in the upper (ad), and in the lower (ab) surface. The mesophyll (assimilation tissue) is with clearly differentiated layers of palisade and spongy parenchyma and is of the dorsoventral type (Fig. 2).

**Fig. 1: General view of plants in areas treated with Isoxaflutole.**

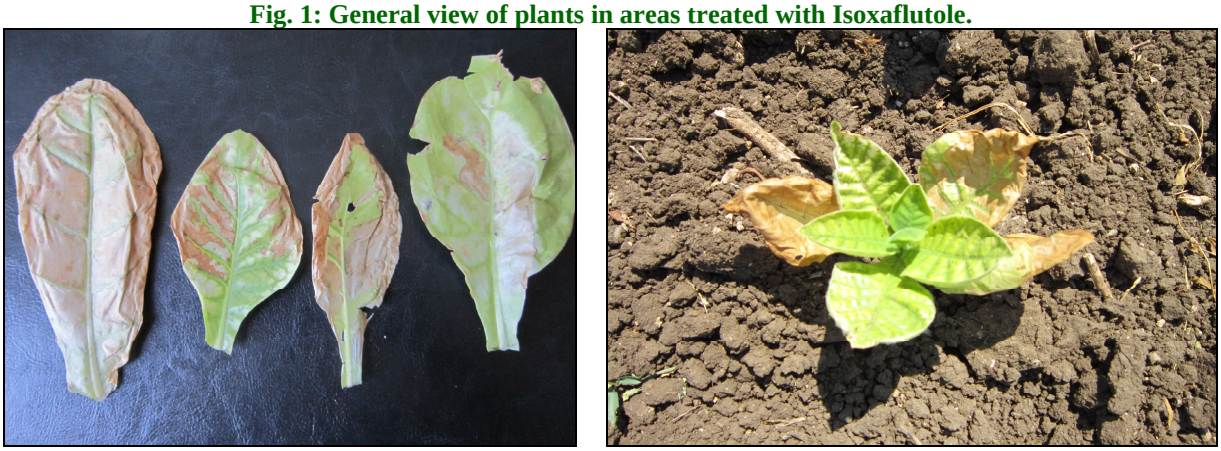
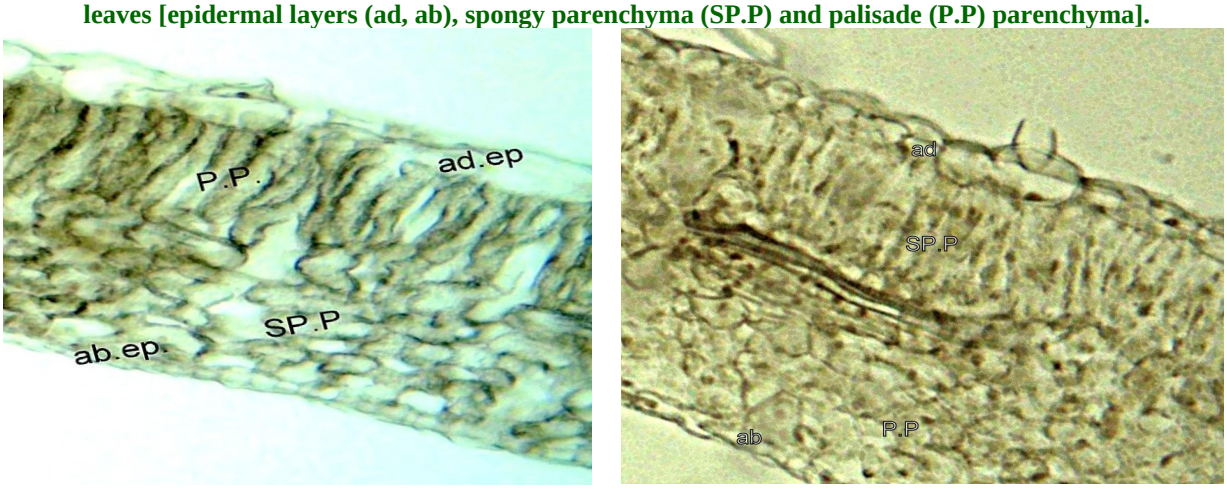


**Fig. 2: Transverse section of *Nicotiana tabacum* leaves. LM, magnification 16×10, (a) control; (b) treated leaves [epidermal layers (ad, ab), spongy parenchyma (SP.P) and palisade (P.P) parenchyma].**




**(a)** **(b)**

The epidermis is composed of basic epidermal, stomata cells and trichomes. The main epidermal cells are arranged disorderly. They are isodiametric in shape and highly curvy anticlinal walls (Figs. 3 and 4). The folds of the anticlinal walls provide greater strength of the epidermis (Georgiev and Chakalova, 1986).

Stomata are positioned on both epidermal surfaces (ad, ab), which defines tobacco leaves as amphistomatic. The number of the supporting cells varies from 3 to 4, and they do not differ from the other basic epidermal cells. These peculiarities define stomata as of the anomocytic type. The guard cells of stomata have a bean-shape form (Figs. 3 and 4). The number of stomata per mm² in untreated leaves (control) in the upper epidermis (ad) was 72.50. According to the resulting coefficient of variation (S%) it can be concluded that they are evenly distributed on the epidermal surface (S% - 11.45). In the samples of the treated plants it was found that their number had drastically decreased - 25.8 per mm², and that their distribution on the upper (ad) surface was uneven, depending on the portions of damage on the tissue. The high coefficient of variation (S%) which was 33.45% is an expression of this distribution of stomata (Table 1). This is the result of established of stomata with very large and very small size (Fig. 3).

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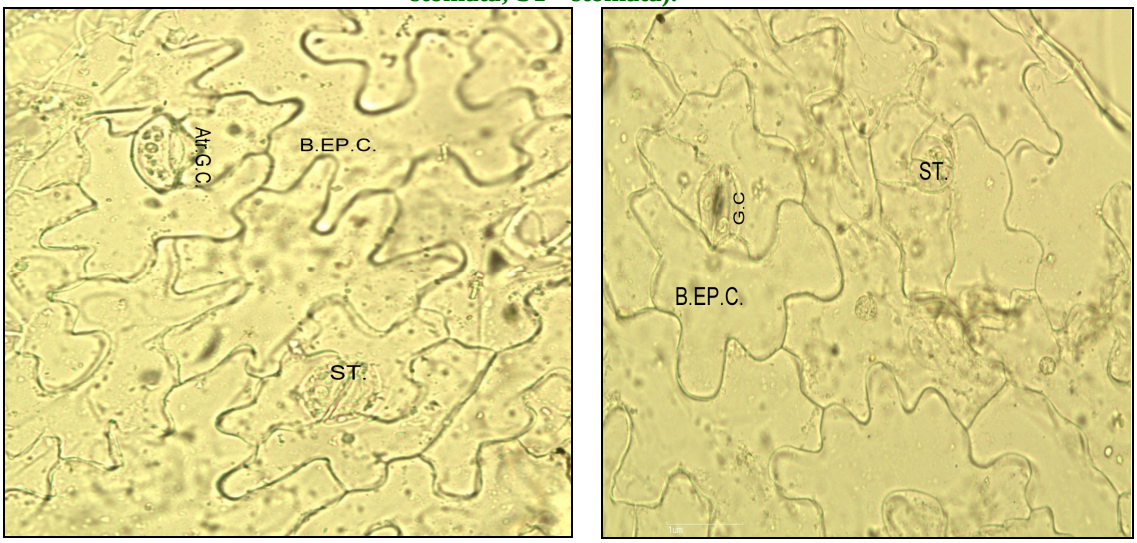
case of the treated plants their number was 43.28% lower (86.25 number/mm²), unevenly situated which is indicated by the high coefficient of variation (S-135.97%) (Table 1). In samples of the leaf in tobacco (*Nicotiana tabacum* L.) from treated areas structural changes were identified in the guard cells of stomata consisting in atrophy of the guard cell, or the stomata themselves are underdeveloped (Fig. 3a).

**Table 1. Morphometric parameters of tobacco leaves (*Nicotiana tabacum* L.)**

| Parameters | | | Average±er. | Std. dev. | S% | max:min |
|---|---|---|---|---|---|---|
| Number of stomata/mm² | 1. Control | ad | 72.50±1.51 | 8.30 | 11.45 | 1.75 |
| | 2. Treated | | 25.83±1.57 | 8.64 | 33.45 | 3.00 |
| | 1. Control | ab | 152.08±2.54 | 13.95 | 9.17 | 1.40 |
| | 2. Treated | | 86.25±21.41 | 117.27 | 135.97 | 18.66 |
| Length stomata/µm | 1. Control | ad | 35.91±0.36 | 2.02 | 5.62 | 1.33 |
| | 2. Treated | | 36.75±1.05 | 5.76 | 15.69 | 2.44 |
| | 1. Control | ab | 35.91±0.38 | 2.12 | 5.91 | 1.30 |
| | 2. Treated | | 34.41±0.98 | 5.40 | 15.69 | 1.8 |
| Width stomata/µm | 1. Control | ad | 27.50±0.31 | 1.73 | 6.31 | 1.20 |
| | 2. Treated | | 27.66±0.61 | 3.34 | 12.08 | 1.44 |
| | 1. Control | ab | 29.66±0.42 | 2.34 | 7.80 | 1.40 |
| | 2. Treated | | 26.33±0.74 | 4.08 | 15.51 | 1.87 |
| Mesophyll thickness/µm | | 1. Control | 239.66±1.69 | 9.27 | 3.87 | 1.13 |
| | | 2. Treated | 189.66±6.35 | 34.78 | 18.34 | 1.92 |

**Fig. 3: Lower surfaces LM, (a) and upper surfaces (b) of the epidermis on the treated leaves *Nicotiana tabacum* with a herbicide (B.ep.c.- basic epidermal cells; Atr. G.c. - atrophy of the guard cells of the stomata; ST - stomata).**

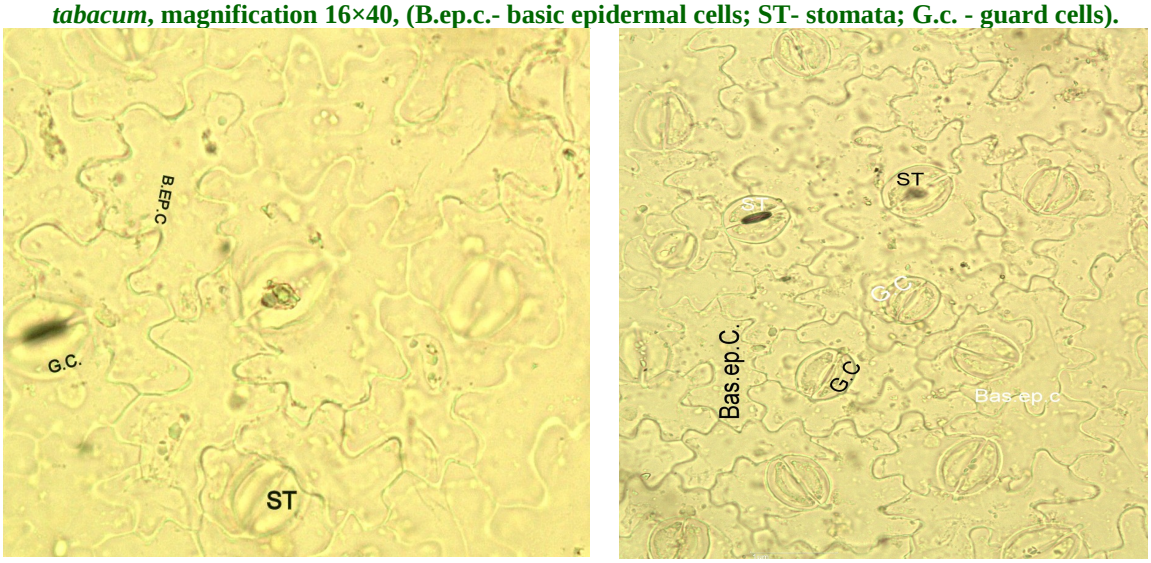



**(a)** **(b)**

Stomata atrophy and the reduction in their number on the epidermal surfaces is the cause of destructive changes in the photosynthetic and gas exchange processes in tobacco plants. The impaired metabolism leads to a decrease in the synthesis of chlorophyll, carotenoids and pigments in the leaves which leads to decreased growth processes in plants. This change in the growth rate correlates with the number of leaves and the height of the stem of tobacco plants and is directly related to the yields obtained (Radoukova and Dyulgerski, 2013).

From the metric measurements it can be seen that the length and width of the guard cells of the stomata vary in the range from 35.91 to 36.75 µm (length), and 27.50 to 29.66 µm (width), the higher values being those of the control samples (Table 1).

**Fig. 4: Lower surfaces LM, (a) and upper surfaces (b) of the epidermis of the control leaves of *Nicotiana tabacum*, magnification 16×40, (B.ep.c.- basic epidermal cells; ST- stomata; G.c. - guard cells).**




**(a)** **(b)**

In the transverse section made it was found that the palisade parenchyma was formed by one layer of cylindrical cells with well-developed chloroplasts (Fig. 2). In the control samples the cells occupy more than 1/3 of the total mesophyll, with intracellular spaces to facilitate the gas exchange processes and photosynthesis in leaves, while in the samples taken from the plants in the treated areas loss of intercellular spaces and density of the structure was observed (Fig. 2b).

The cells constituting the spongy parenchyma are oval, located in 7-8 rows, with more or less small intracellular spaces in the control and without spaces, highly compacted, in the plants treated with herbicide. The morphometric analysis of the measurement of the total thickness of the mesophyll shows that for the control it was 236.66 μm, while for the plants treated with herbicide it was reduced to 189.66 μm. This reduction in the thickness of the assimilation parenchyma is 20% and is directly related to the chlorosis found, to the deformation of the leaf blade and the inhibition of the growth processes in the plants. These changes were confirmed also by the resulting high coefficient of variation in the samples from the treated areas - 18.34%, which proves the deviations from mean values. This is not by accident, since variations were found in the sections of the leaf blade with lesions (necrosis) and with preserved structure.

Leaf veins are collateral, composed of adaxially positioned xylem and abaxially situated phloem. The anatomical features of tobacco leaves (*Nicotiana tabacum* L) found are consistent with has been established by Nikitin and Pankova (1982) and according to a classification proposed by Donev et al. (1981), the structure of the leaf plate established by us can be attributed to the tobacco group with mesophytic structure of the leaves.

## Conclusion

The Isoxaflutole cause adversely affects in tobacco plants (*Nicotiana tabacum* L.).The morphological manifestations of phytotoxicity are expressed in chlorosis, growth inhibition and necrosis of the leaves. The structural and anatomical changes are related to a reduced number of stomata, both on the upper and lower epidermis. Also, atrophy of the guard cells of the stomata and reduction of the thickness of the assimilation parenchyma.

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