

Preliminary biochemical studies on a model growing of different tobacco plant (*Nicotiana tabacum* L.) cultivars

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Summary: The aim of our preliminary studies was to characterize the change in the activity of enzymes PPO and POX, the concentration of total soluble phenolic compounds and soluble protein content of different tobacco cultivars measured in tobacco plants during cultivation. The results suggest that a shorter cultivation period (13–14 weeks) is more favourable for tobacco plants as protein source than for tobacco industrial use (16–17 weeks). We found some correlation between the concentration of total soluble phenolic compounds and PPO activity data. In the increasing and maximum period of total phenol content the PPO activity was high. But later because of decrease of substrate phenol content the activity of PPO also decreased. We found higher soluble protein concentrations in Virginia than in Burley varieties.

Key words: peroxidase, polyphenol oxidase, protein source, soluble protein content, tobacco cultivation, total phenol content

Introduction

Tobacco plant (*Nicotiana tabacum* L.) is one of the most studied materials. It is used not only in the tobacco industry but also for other purposes. Nicotine in isolated form is a component of sprays in plant protection and this compound is an important agent in the study of different nerve-cell receptors. Leaf proteins, among them tobacco leaf proteins, are essential protein sources (Tso, 1990).

Polyphenols and pigments are important components influencing tobacco quality and usability (Tso, 1990). One of these phenol derivatives catechol (1,2-dihydroxybenzene), an active carcinogenic agent can be produced by pyrolysis via smoking (Schlotzhauer *et al.*, 1982). It was presumed that the study of the activity of the enzymes taking part in the metabolism of polyphenols and pigments could be informative about the quality of tobacco plant and products. The total phenol content (phenol and dihydroxybenzol derivatives e.g. chlorogenic acid, flavonoids, etc.) of tobacco is synthesized generally by way of deamination of aromatic amino acids phenylalanine and tyrosine via phenylpropanoid pathway in tobacco (Tso, 1990). The concentration of phenol derivatives in the tobacco plant is determined by the balance of their synthesis and degradation.

Polyphenol oxidases (PPO) (EC 1.14.18.1) are copper enzymes catalyzing the oxidative degradation of dihydroxybenzol (pyrocatechol and hydroquinon) derivatives (synthesized by the oxidation of phenol content) to quinones by molecular oxygen. These quinones can polymerize spontaneously to different pigments (e.g. melanines). These

processes can be useful in tobacco (by enrichment in colour and quality agents). On the other hand it can cause a deterioration in nutrient quality of fruits and vegetables (by enzymatic browning) (Shi *et al.*, 2002).

In the case of various stress conditions (e.g. boron deficiency or draft) not only the key enzymes in the biosynthesis of phenolic compounds were activated but the activity of PPO was also enhanced. In the accumulation and oxidation of different polyphenol derivatives undesirable toxic active oxygen species could form, therefore the activity of enzymes, above all peroxidases (POX) (EC 1.11.1.7), for the degradation of these dangerous species was also increased (Camacho-Cristóbal *et al.*, 2002).

Tobacco leaves as well as other higher plant leaves contain soluble and insoluble proteins which are usually equal in quantity (Tso, 2006). Tobacco leaf proteins are well balanced containing high levels of essential amino acids. Nowadays high protein content, especially soluble protein fraction (F1 and F2 proteins) of the tobacco plant is considered a protein source not only in animal husbandry but also as supplements in humans nutrition (Kung *et al.*, 1980). It is possible to isolate soluble proteins and at the same time make use of leaf material for smoking.

The aim of our preliminary studies was to characterize the change in the activity of enzymes PPO and POX, the concentration of total soluble phenolic compounds and soluble protein content of different tobacco cultivars measured in tobacco plants during cultivation. It is known that tobacco is especially sensitive to agronomic practices (Ruggerio *et al.*, 2004), therefore during our studies we made

efforts to maintain almost the same conditions for all of plants examined.

Material and methods

Materials

Two varieties of both Burley (B-TMC1, B-TMC2 and B-TMC3) and Virginia (V-TMC1, V-TMC2 and V-TMC3) tobacco cultivars (*Nicotiana tabacum* L.) (V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary) were grown in Budapest (Hungary). Tobacco seedlings were grown in a seedbed by V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary then we transplanted them to individual plastic plant bags (15 l) containing a soil mixture (Palántaföld) of different kinds of peats and composts (GARRI Trade and Service Ltd., Budapest, Hungary) on June 2nd 2005 then they were grown under open-air conditions. Apart from everyday watering (except rainy days) the soil was treated with a complex artificial fertilizer with nitrogen content 15%, phosphorous content 15% and potassium content 15% (GRAN-EXPORT Ltd., Debrecen, Hungary) (50 g m⁻²) on July 13th, July 25th, August 9th, August 18th and August 22nd 2005. From August 10th until August 23rd samplings of leaves (both upper-leaves and bottom-leaves separately) were taken the day after the fertilizations and then on August 31st, September 6th, September 15th, September 23rd, until the harvest that took place on September 29th 2005. The tobacco leaves were homogenized and the extracts (0.10 g ml⁻¹ in water after centrifugation for 10 min at 4 °C at 10,000 rpm) were made. Chemicals were purchased from SIGMA-ALDRICH Co. and REANAL Finechemical Co. (Budapest, Hungary).

Methods

POX activity of the extracts was determined using *o*-dianisidine as hydrogen donor in sodium acetate (pH 5.1) (Bjorkstein, 1968). One unit of POX activity was defined as the amount of enzyme that caused decomposition of 1 mole hydrogen peroxide in the reaction mixture (1.0 ml) in 1 min.

PPO activity was detected in sodium acetate (instead of sodium phosphate) buffer (pH 6.5) using pyrocatechol substrate (Watson & Flurkey, 1986). One unit of PPO activity was defined as the amount of enzyme that caused 0.01 unit of change in absorbance of the reaction mixture (1.0 ml) in 1 min at 420 nm.

The concentration of total soluble phenolic compounds was measured by the colorimetric method with Folin & Ciocalteu's phenol reagent (Singleton & Rossi, 1965) and the results were expressed in Gallic Acid Equivalent (GAE) value (mmol gallic acid dry weight of tobacco leaves g⁻¹).

Soluble protein concentration was measured by Layne method (Layne, 1957). Data of samples were calculated on the dry weight of leaves. Water content of the samples were determined by Sartorius M 50 Aquatest instrument. The

results are given as the average values of four replications with a standard deviation 5%.

Results and Discussion

Two varieties of both Virginia (V-TMC1, V-TMC2, V-TMC3) and Burley (B-TMC1, B-TMC2, B-TMC3) tobacco cultivars (*Nicotiana tabacum* L.) (V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary) were used. These variety-names are artificial names given by the business-management of the Company in these double blind tests. The cultivation of these varieties is widely distributed in Hungary.

Earlier complex extraction methods were generally used by Tris-acetate buffer and surfactants then after more or less isolation followed by the analyses of tobacco leaves (Ruiz et al., 1998). But earlier we found that because of the presence of the hydrophobic components in food industrial raw materials of plant origin a concentrated aqueous solution (500 g L⁻¹ in water) could be used for the measurement of the activity of enzymes with a pH optimum lower than pH 7.5 and the concentration of total soluble phenolic compounds of the samples without previous isolation (Kápolna et al., 2006). Because of the relatively low water content of tobacco plant (lower than in fruits) aqueous solutions were only 100 g L⁻¹ in water. Data measured of concentrated aqueous extractions were compared to data of other extractive agents described in the literature and no significant differences were found.

The changes of phenol derivatives in tobacco leaves during flue-curing process were studied earlier. A rapid increase was found in the first 24 h of curing then a decrease was detected until 72 h. At the end of curing again an increase was measured. The changes in PPO and POX activity were the opposite (Gong et al., 2006).

In the concentration of total soluble phenolic compounds expressed in GAE value (mmol gallic acid dry tobacco g⁻¹) of tobacco leaves we found an enhance till 23.08, then a decrease until a quasi-constant level (0.3–0.4) (Figures 1–4). This was attributed to the balance of the synthesis of phenols and then *via* their degradation the synthesis of colouring agents. We found higher values (0.80–1.0 for Virginia and 1.0–1.4 for Burley varieties) in upper leaves (the younger) than bottom leaves (the older) (0.70–1.0 for Virginia and 0.7–0.9 for Burley varieties). The high maximum total phenol levels in Burley varieties upper leaves suggests a more intensive synthesis of colouring agents than in Virginia varieties.

In PPO activity (U dry tobacco g⁻¹) of tobacco leaves we found a high activity (18000–50000 for Virginia and 12000–40000 for Burley varieties in upper leaves; 12000–13000 for Virginia and 12000–16000 for Burley varieties in bottom leaves) at the start of our studies then a significant decrease in upper leaves (1900–8400 for Virginia and 7300–14000 for Burley varieties) and a radical decrease (80–100 for Virginia and 90–100 for Burley varieties) in bottom leaves (Figures 5–8). We found some correlation between the concentration of total soluble phenolic compounds and PPO activity data. In the increasing and ma-

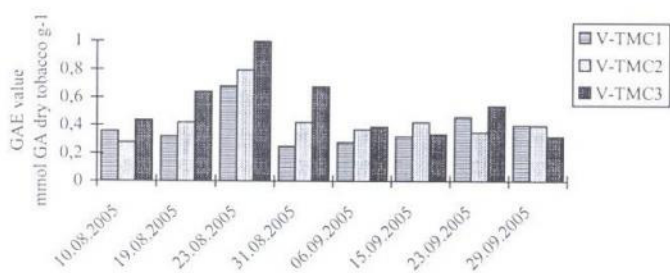


Figure 1 Changes in the concentration of total soluble phenolic compounds expressed in GAE value (mmol gallic acid dry tobacco g⁻¹) in upper leaves of different Virginia tobacco varieties during cultivation

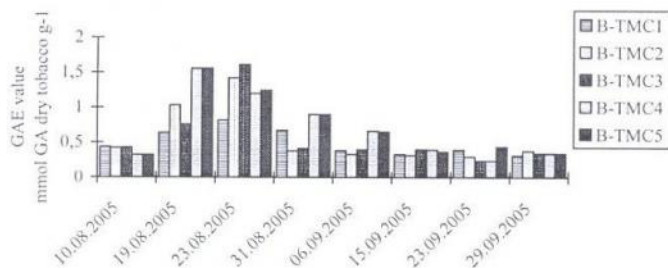


Figure 2 Changes in the concentration of total soluble phenolic compounds expressed in GAE value (mmol gallic acid dry tobacco g⁻¹) in upper leaves of different Burley tobacco varieties during cultivation

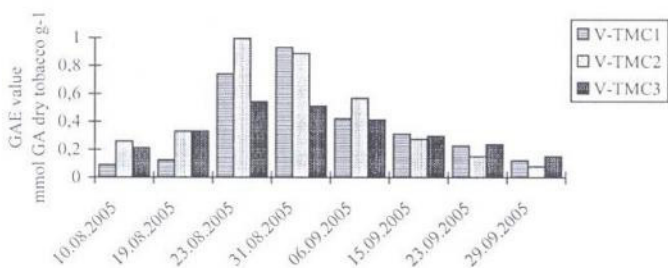


Figure 3 Changes in the concentration of total soluble phenolic compounds expressed in GAE value (mmol gallic acid dry tobacco g⁻¹) in bottom leaves of different Virginia tobacco varieties during cultivation

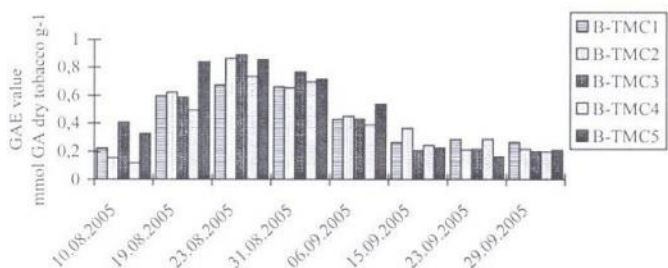


Figure 4 Changes in the concentration of total soluble phenolic compounds expressed in GAE value (mmol gallic acid dry tobacco g⁻¹) in bottom leaves of different Burley tobacco varieties during cultivation

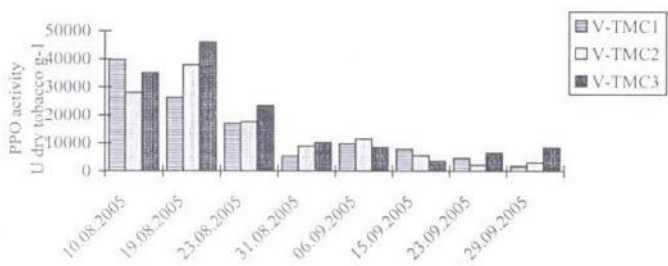


Figure 5 Changes in PPO activity (U dry tobacco g⁻¹) in upper leaves of different Virginia tobacco varieties during cultivation

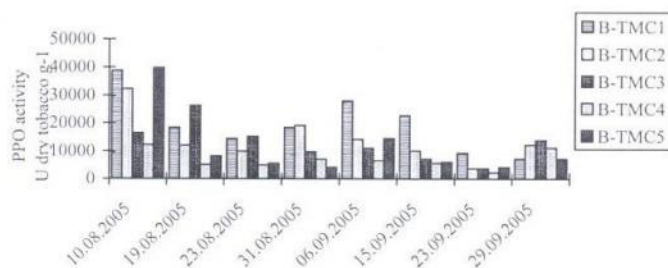


Figure 6 Changes in PPO activity (U dry tobacco g⁻¹) in upper leaves of different Burley tobacco varieties during cultivation

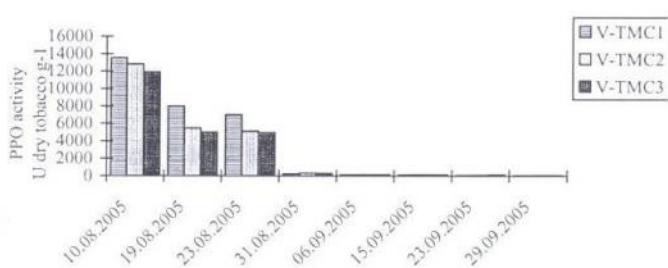


Figure 7 Changes in PPO activity (U g dry tobacco g⁻¹) in bottom leaves of different Virginia tobacco varieties during cultivation

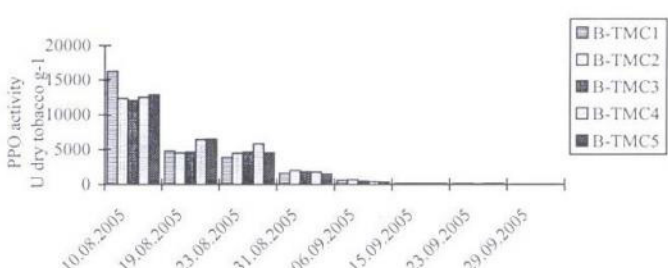


Figure 8 Changes in PPO activity (U dry tobacco g⁻¹) in bottom leaves of different Burley tobacco varieties during cultivation

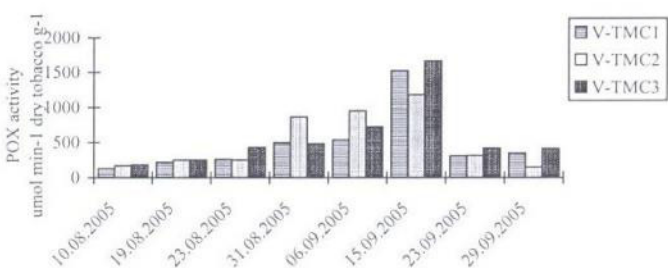


Figure 9 Changes in POX activity (umol min⁻¹ dry tobacco g⁻¹) in upper leaves of different Virginia tobacco varieties during cultivation

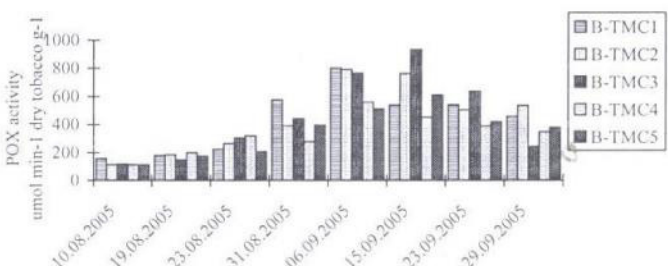


Figure 10 Changes in POX activity (umol min⁻¹ dry tobacco g⁻¹) in upper leaves of different Burley tobacco varieties during cultivation

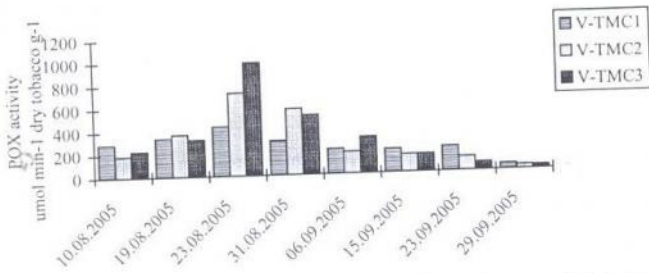


Figure 11 Changes in POX activity ($\mu\text{mol min}^{-1}$ dry tobacco g^{-1}) in bottom leaves of different Virginia tobacco varieties during cultivation

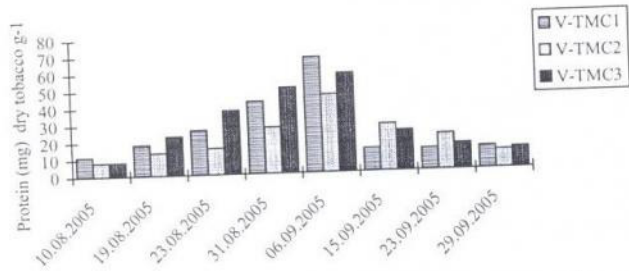


Figure 13 Changes in soluble protein content (mg protein dry tobacco g^{-1}) in upper leaves of different Virginia tobacco varieties during cultivation

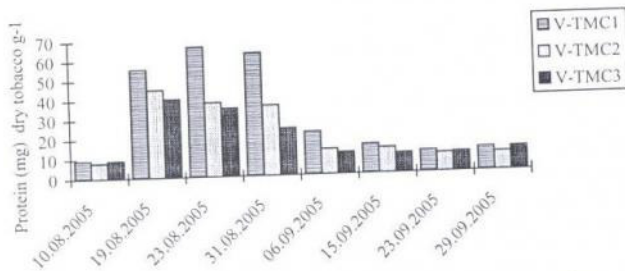


Figure 15 Changes in soluble protein content (mg protein dry tobacco g^{-1}) in bottom leaves of different Virginia tobacco varieties during cultivation

ximum period of the total phenol content the PPO activity was high. But later because of decrease of substrate phenol content the activity of PPO also decreased. The reduction in enzyme activity was higher in bottom leaves (the older) than in upper leaves (the younger).

The tendency in changes of POX activity ($\mu\text{mol min}^{-1}$ dry tobacco g^{-1}) of tobacco leaves was deviated from the results of total phenol content and PPO activity and could be in connection with the age of the plant (Figures 9–12). We found the maximum values earlier in bottom leaves (23.08 for both in Virginia and Burley varieties) than in upper leaves (15.09 in Virginia and 06.09 in Burley varieties). The maximum data of upper leaves were higher in Virginia (1500–1700) than in Burley (500–800) varieties and almost the same in bottom leaves (400–1000).

In the increasing and maximum period of soluble protein content of tobacco leaves (mg protein dry tobacco g^{-1}) of various Virginia and Burley varieties the dispersion of data measured was slightly higher and maximum period was slightly later (06.09) for upper leaves and longer (19.08–31.08) for bottom leaves both in Virginia and Burley varieties than in the case of other parameters (Figures 13–16). The maximum

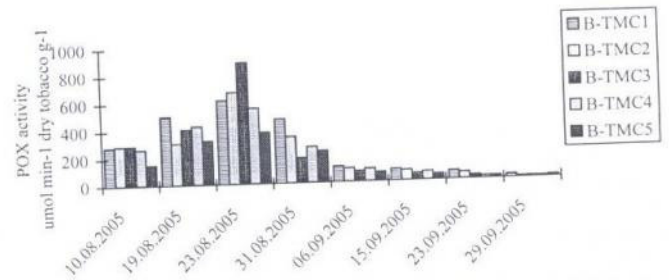


Figure 12 Changes in POX activity ($\mu\text{mol min}^{-1}$ dry tobacco g^{-1}) in bottom leaves of different Burley tobacco varieties during cultivation

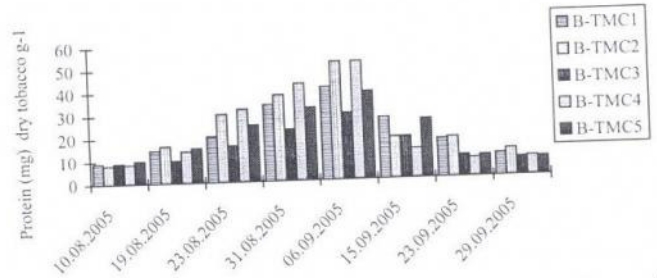


Figure 14 Changes in soluble protein content (mg protein dry tobacco g^{-1}) in upper leaves of different Burley tobacco varieties during cultivation

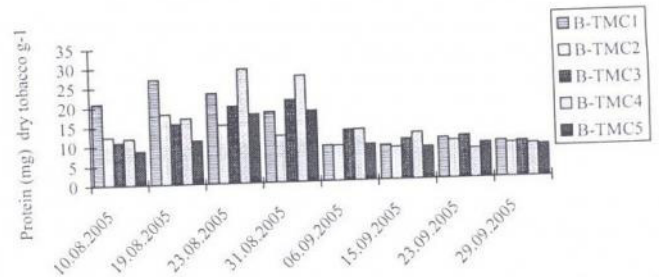


Figure 16 Changes in soluble protein content (mg protein dry tobacco g^{-1}) in bottom leaves of different Burley tobacco varieties during cultivation

data of both upper and bottom leaves were higher in Virginia (46–69 for upper and 40–50 for bottom leaves) than in Burley (29–52 for upper and 18–29 for bottom leaves) varieties. The quasi-constant level was also higher in Virginia (10–15) than in Burley (7–12) varieties both in upper and bottom leaves. These results suggest that a shorter cultivation period (13–14 weeks) is more favourable for tobacco plants as protein source than for tobacco cultivated for industrial use (16–17 weeks).

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