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# Response of Gas Exchange to Leaf Piercing Explained by Piecewise Linear Regression for Two Developmental Forms of Rape Plant (*Brassica napus* L. ssp. *oleifera* Metzg)

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Oilseed rape (*Brassica napus* L. ssp. *oleifera* Metzg) was the subject of the study in two forms: winter *cv*. 'Muller' (at the rosette stage – the first internode BBCH 30 – 31) and spring *cv*. 'Feliks' (at the yellow bud stage BBCH 59). The main gas-exchange parameters, net photosynthetic rate ($P_N$), transpiration rate ($E$), stomatal conductance ($g_s$), and intercellular $CO_2$ concentration ($C_i$) were measured on leaves prior to the piercing and immediately after the short-term piercing. The effect of mechanical wounding revealed different progress of the gas exchange process for the two forms. Piecewise linear regression with the breakpoint estimation showed that the plants at the same age but at a different vegetal stage, manage mechanical leaf-piercing differently. The differences concerned the stomatal conductance and transpiration changes since for rosette leaves the process consisted of five intervals with a uniform direction, while for stem leaves - of five intervals with a fluctuating direction. These parameters got stabilized within a similar time (220 mins) for both forms. The process of net photosynthetic rate was altered by the plant stages. 'Muller' plants at the rosette stage demonstrated dependence of $P_N$ on time in *log*-linear progression: $y\ (P_N) = 8.01 + 2.73 \log_{10} (x\ t_2)$; $7 < t_2 < 220$; $R^2 = 0.96$. For stem leaves of 'Feliks' plants the process of transpiration, in terms of directions, was convergent with the process of photosynthesis. Those two processes were synchronized from 1st to 114th min of the test ($r = 0.85$; $p < 0.001$) in plants at the rosette stage and from 26th to 148th min in stem leaves ($r = 0.95$; $p < 0.001$).

**Keywords:** leaf piercing response, oilseed rape, net photosynthetic rate, transpiration, stomatal conductance, concentration of intercellular $CO_2$

**Abbreviations:**
$C_i$ – intercellular $CO_2$ concentration
CCI – chlorophyll content index
$E$ – transpiration rate
$g_s$ – stomatal conductance
LA – leaf area
$P_N$ – net photosynthetic rate

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## INTRODUCTION

Biomass production mostly depends on the level of gas exchange between the plant and the environment. Stressful environments, including drought, salinity, and unfavorable temperatures, considerably hamper the process of photosynthesis in most plants by altering the ultrastructure of the organelles and concentration of various pigments and metabolites including enzymes involved in this process as well as stomatal regulation (Ashraf and Harris, 2013). Plant respiration can be disturbed by biotic and abiotic stressors (Roitsch, 1999). The appearance of the stress factor caused by, e.g., hailstorm (Muro et al., 1998; Tartachnyk and Blanke, 2002) or an invasion of plant-eating pests (Hawkins et al., 1987; Holman and Oosterhuis, 1999; Gomez et al., 2004) results in stomata closing, decreasing the intensity of photosynthesis, sometimes also increasing evaporation from leaves. Changes in the intensity of gas exchange occur immediately after the induction of stress reaction. The response to stress is detectable by the net photosynthetic rate as well as transpiration and mostly depends on the plant species and the size of the leaves left on the plant after defoliation as well as the presence or lack of the apical bud (Evans, 1991; Wang et al., 1997; Roitsch, 1999). Despite many various types of damage triggered by biotic factors (insects, viruses, fungi), for various host plants the mechanism of regulation is the same and it involves a decrease in the transcription of nuclear genes encoding the components of photosynthesis. Biotic leaf wounding causes almost complete inhibition of genes taking part in the process of photosynthesis. It is seen mostly for the genes connected with the synthesis of pigment and transport of electrons (Bilgin et al., 2010). After infection caused by pathogens or plant-eating insects the effort of the plant to decrease the amount of photosynthetic protein is necessary to support the defense induction. The leaf nitrogen is involved in; much of it is found in photosynthetic proteins, primarily in RuBisCO, and if limited, it can even lead to its use with RuBisCO (Paul and Foyer, 2001). The infestation of scale insects decreases the chlorophyll and carotenoid content as well as the value of three indicators of photosynthetic activity. The reactions depend on the specific properties of plants and abundance of insects feeding on them (Golan et al., 2015). The research into rape (*Brassica napus* var. *oleifera*) performed so far demonstrated that beetles feeding on pollen (*Meligethes aeneus*) decrease the activity of the photosynthetic apparatus or increase transpiration, through a clear decrease in photosynthesis in unprotected plants, high stomatal conductance and poor fixation of $CO_2$. It is also known, as for that species, that the wounding compensation methods are high effective (Axelsen and Nielsen, 1990) and mostly result from its high genetic potential which facilitates the formation of a huge number of flower buds, namely about 4-5 thousand per plant. The rape plants artificially deprived of inflorescence on the main shoot demonstrate a photosynthesis compensation capacity, optimizing the parameters of gas exchange; they decrease stomatal conductance.

The effect of mechanical stress on leaves is ambiguous for photosynthesis when the following conditions are considered: the plant species and age, as well as the stress type and its duration (Biddington, 1984). It was confirmed by Blamowski et al. (2003) for two species representing the genus *Brassica*: radish and spring rape at the rosette stage, exposed to the same wounding, i.e., the oldest leaves removal or defoliation of the youngest ones with the apical stem. Stress affects the course of gas exchange, distribution of assimilates and plant growth. Moreover, each interference in the relationship and cooperation of the organs providing the source of recipients of organic compounds also disturbs the production and activity of growth regulators. The compounds can regulate the distribution of assimilates, growth and gas exchange by affecting the biosynthesis and activity of enzymes or the absorption of gases (Starck and Ubysz, 1976; Pinto, 1980).

The aim of the present research was to determine the effect of mechanical wounding caused by short-term piercing of leaves in rape in its two developmental forms: the winter form at the rosette stage – the first internode (BBCH 30–31) and spring rape plant at the yellow bud stage (BBCH 59), on the progress of variation in the processes of assimilation and transpiration as well as stomatal conductance and the content of intercellular $CO_2$. We hypothesized that the leaves of the two cultivars (spring / winter form) belonged to *B. napus* sp., and because they came from plants at the same age but at different developmental stages (generative / vegetative), they represented various course of gas exchanges after a short leaf piercing.

## MATERIAL AND METHODS

### PLANTS AND TREATMENT

Oilseed rape (*Brassica napus* L. ssp. *oleifera* Metzg) was the subject of the study in two forms: winter *cv*. 'Muller' and spring *cv*. 'Felix'. Seeds obtained from a breeding company were germinated and grown in peat-filled pots (15 cm × 15 cm × 20 cm) in a greenhouse at 9/3°C (day/night environment) during the spring of 2014. Watering was applied to maintain the moisture at 65% relative water content. After 21 days, these plants at 4–5 leaf stage (BBCH 14–15) were transferred

to a chamber at 20/11°C (day/night) for 10 days, received 300 µmol $m^{-2}$ $s^{-1}$ light for 14 h per day, the relative humidity was 50%. Fertigation was applied according to the scheme with macronutrients (g $dm^{-1}$ of nutrient solution): 2.0 $Ca(NO_3)_2 \cdot 4H_2O$ (calcium nitrate tetrahydrate), 1.5 $KNO_3$ (potassium nitrate), 0.75 $(NH_4)_2SO_4$ (ammonium sulfate), 0.55 $MgSO_4 \cdot 7H_2O$ (magnesium sulfate heptahydrate), 0.35 $KH_2PO_4$ (monopotassium phosphate) and for micronutrients (g $dm^{-1}$ of nutrient solution): 0.33 Cu IDHA, 2.0 Mn IDHA, 0.57 Zn IDHA, 0.28 $H_3BO_3$, EC = 2.6 and pH = 5.6. Application of nutrients was done twice a day with two emitters per pot dropping 100 ml for 60 s (Ferdiga system). Rape plants in 60 pots (30 per each cultivar) were cultivated until they reached BBCH 30–31 (rosette – the first internode) – *cv.* 'Muller', and BBCH 59 (the first petals 'yellow bud') – *cv*. 'Feliks' plants. All plants were the same age, however, their varied development was due to the fact that *cv*. 'Muller', as a winter form, remained at the rosette (vegetative) stage, while *cv*. 'Feliks', as a spring form, started the generative stage. The treated, fully expanded third leaves were chosen with the uniform area and CCI (measured by the chlorophyll meter CCM-200 plus, Opti-Sciences, Inc., USA) – Table 1. Wounding by piercing of the total leaf area with the pins having a diameter of 1 mm and a density of 10 punches per 1 $cm^2$ lasted 3 seconds.

### MEASUREMENTS

The main gas-exchange parameters, net photosynthetic rate ($P_N$), transpiration rate ($E$), stomatal conductance ($g_s$), and intercellular $CO_2$ concentration ($C_i$) were measured prior to the piercing and immediately after the piercing at the center of the wounded area, the leaves were fitted into a 6.25 $cm^2$ clamp-on Plant Leaf Chamber (PLC Broad with mixed Red/Blue LED array). Gas exchange measurements lasted 220 mins. This was performed using a portable open infrared $CO_2$ gas analyzer (LC-Pro+, ADC BioScientific Ltd, Hoddesdon, UK) between 10:00–14:00 h. The system allowed for an automated microclimate control in the PLC. The conditions were stable in PLC and amounted to light 600 µmol $m^{-2}$ $s^{-1}$, $CO_2$ concentration 360 ± 5 ppm; temperature 22 ± 1°C, water vapor pressure 10 ± 1 mbar (relative humidity approx. 40%). The rate of air flow through the LCpro+ chamber was approximately 200 ml·$min^{-1}$. These conditions provided the strongest response of plants to leaf piercing. In the growth chamber where the gas-exchange parameters were measured oxygen concentration was ambient (21%).

### DATA ANALYSIS

The analysis of piecewise linear regression with the breakpoint estimation was calculated to explain the time-relation changes of the parameters $g_s$, $C_i$, $P_N$, and $E$. The time intervals were estimated by nonlinear methods according to Quasi-Newton and the lost function based on the least squares were proceeded (Haelterman et al., 2009). The relationships between the parameters were computed using the simple coefficient of correlation ($r$ by Pearson). The results were processed using STATISTICA data analysis software system version 12.0 (StatSoft; Tulsa, Oklahoma, USA).

## RESULTS

The characteristics of leaves are presented in Table 1. Both cultivars represented uniform LA ($F_{(1;58)} = 2.59$, $p = 0.12$), and the CCI was also statistically insignificant ($F_{(1;58)} = 3.44$, $p = 0.07$). The response to leaf piercing with pins was noticeable in both cultivars, however, its pattern varied in terms of the intensity of gas exchange parameters in time and in relationships between them.

### STOMATAL CONDUCTANCE AND INTERCELLULAR CARBON DIOXIDE CONCENTRATION AS RESPONSE TO LEAF PIERCING

Stomatal conductance before piercing amounted to 0.280 mol $m^{-2}s^{-1}$ in 'Muller' winter rape plans and 0.390 mol $m^{-2}s^{-1}$ in 'Felix' spring rape (Table 2). Upon leaf piercing, $g_{s1}$ decreased in 'Muller' by 0.01 mol $m^{-2}s^{-1}$ within 1 min, i.e., by 26% over 5.7 mins and in 'Feliks' it decreased by 0.006 mol $m^{-2}s^{-1}$, i.e., by 7% over 5.9 mins (Table 2). After the first interval $g_{s2}$ increased

TABLE 1. The characteristics of leaf of rape cultivars.

| Characteristic | Cultivar/stage | | $F_{(1;58)}$ | $p$ |
|---|---|---|---|---|
| | 'Muller' BBCH 30–31 | 'Feliks' BBCH 59 | | |
| LA (c = 0.8) | 49.6 ± 2.47 | 44.2 ± 2.30 | 2.59 | 0.12 |
| CCI | 21.13 ± 1.36 | 17.65 ± 1.29 | 3.44 | 0.07 |

TABLE 2. Progress of stomatal conductance [$g_s$] changes after leaf piercing of two rape cultivars according to the piecewise linear regressions of the time and with confident limits (CL).

| Break point of time [min] | 95 % CL of time | $g_s$ [mol m$^{-2}$ s$^{-1}$] | 95 % CL of $g_s$ | Relative stepwise change [%] |
|---|---|---|---|---|
| *cv* 'Muller' BBCH 30-31 | | | | |
| Starting = 0.0 | – | 0.280 | – | 100 |
| 5.7 | 5.5–5.9 | 0.207 | 0.190–0.220 | - 26 |
| 27.4 | 25.3–29.5 | 0.242 | 0.238–0.246 | + 17 |
| 58.5 | 54.7–62.4 | 0.246 | 0.243–0.250 | + 1.7 |
| 116.0 | 110–122 | 0.294 | 0.289–0.300 | + 20 |
| Ending = 220 | – | 0.354 | – | + 20 |
| *cv* 'Feliks' BBCH 59 | | | | |
| Starting = 0.0 | – | 0.390 | – | 100 |
| 5.9 | 5.0–6.8 | 0.361 | 0.359–0.363 | - 7 |
| 23.6 | 20.9–26.5 | 0.384 | 0.376–0.391 | + 6 |
| 63.4 | 60.4–66.4 | 0.265 | 0.261–0.269 | - 29 |
| 155.0 | 149–161 | 0.408 | 0.407–0.409 | + 48 |
| Ending = 220 | – | 0.421 | – | + 4 |

by 0.002 mol m$^{-2}$s$^{-1}$ per 1 min (17%) over 6 – 27.4 mins in 'Muller' and by 0.0013 mol m$^{-2}$s$^{-1}$ (6%) over 6 – 23.6 mins in 'Feliks' (Figs. 1a, 1b). Over the next interval there was seen a diametrically opposite reaction of $g_s$ between winter and spring plants; 'Muller' was going through a 30 mins period of uniform level $g_{s3}$ of 0.246 mol m$^{-2}$s$^{-1}$, whereas in 'Feliks' $g_{s3}$ was decreasing for 40 mins, every minute by 0.0028 mol m$^{-2}$s$^{-1}$, i.e., by 29%, to the level of 0.265 mol m$^{-2}$s$^{-1}$ (Table 2). Only about an hour after piercing, the plants of both rape plant forms started the period of $g_{s4}$ stabilization (Figs. 1a, 1b). In the 'Muller' plants, between 59$^{th}$ and 116$^{th}$ min, $g_{s4}$ increased by 0.001 mol m$^{-2}$s$^{-1}$, i.e., by 20%, and during successive 100 mins ($g_{s5}$) also by 20%, at the rate of 0.0004 mol m$^{-2}$s$^{-1}$. In the 'Feliks' plants,

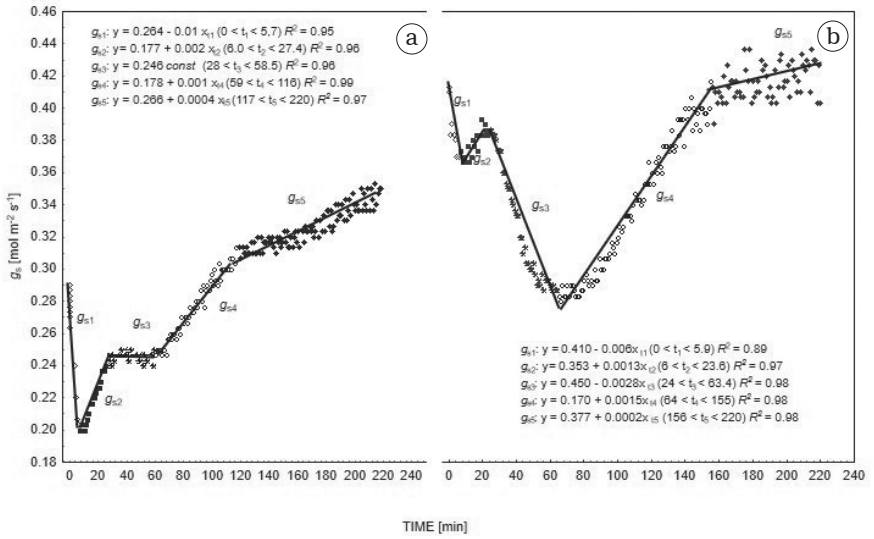


**Fig. 1**. Piecewise linear regressions for stomatal conductance after leaf piercing of rape plant. The confident limits (95% CL) of the linear parameters, the duration of each interval (t) and coefficients of determination ($R^2$); (**a**) *cv.* 'Muller', (**b**) *cv.* 'Felix'.

between 63rd and 159th min, $g_{s4}$ increased by 48% at the rate of 0.0015 mol $m^{-2}s^{-1}$ per 1 min, and for successive 65 mins ($g_{s5}$) – by 4% at the rate of 0.0002 mol $m^{-2}s^{-1}$ per 1 min (Table 2, Figs. 1a, 1b).

Prior to the piercing, the concentrations of intercellular $CO_2$ ($C_i$) were 211 and 214 µmol $mol^{-1}$ for 'Muller' and 'Feliks', respectively (Table 4). Two minutes after piercing $C_{i1}$ increased by 22 µmol $mol^{-1}$ (11%) in 'Feliks' and by 47 µmol $mol^{-1}$ (22%) in 'Muller' over 3 mins (Table 4). An hour after the piercing, $C_i$ displayed a similar tendency to increase together with the $g_s$ increase in both cultivars. After the first interval of growth there followed two periods of decrease in $C_{i2}$ and $C_{i3}$; $C_{i2}$ at a higher rate (-1.77 and -1.40 µmol $mol^{-1}$ for 'Muller' and 'Feliks', respectively) and $C_{i3}$ – at a slower rate (-0.42 and -0.18 µmol $mol^{-1}$ for 'Muller' and 'Feliks', respectively). Such response lasted up to 46–64 mins after piercing, which was followed by the stage of $C_{i4}$ growth in both cultivars at the same rate (by 0.11 µmol $mol^{-1}$ every minute) taking from 46th to 114th min in 'Muller' and from 63.5th to 146th min in 'Feliks', and then the $C_{i5}$ period of stabilization (by 0.02 µmol $mol^{-1}$ in 'Feliks' and 0.05 µmol $mol^{-1}$ in 'Muller', every minute) – from 146th to 220th min and from 114th to 220th min of the test, respectively (Table 4, Figs. 2a, 2b).

Changes in $g_s$ in 5 time sequences can be interpreted by the relationships between $C_i$ and transpiration ($E$), and net photosynthetic rate ($P_N$), calculated as *r*-Pearson correlation coefficient and *b* coefficient of regression (Table 3). Provided that the power and direction of the correlations between $g_s$ *vs.* $E$ and $P_N$ were similar for both rape cultivars after piercing stress in 5 time intervals and throughout the test, the relationship between $g_s$ and $C_i$ in the case of 'Muller' was different from that in 'Feliks'. In 'Feliks' it was a strong positive correlation between $C_i$ with $g_s$ ($r$ = 0.86), while in 'Muller' – negative correlation with $r$ = -0.81, which means that the leaves at the rosette stage retained $CO_2$ during the decrease in $g_{s1,2}$ over 1st–23rd min, while the leaves at the flowering stage were losing intercellular $CO_2$ with a decrease in $g_{s1,3}$ (from 1st to 6th min and from 24th to 63rd min) (Table 3, Figs. 2a, 2b). That proves the different initial response to the piercing. An hour after piercing the tendency in both cultivars got leveled off and resulted in an increase in $C_{i4}$ by 10.2 ('Muller') and 7.78 ('Feliks') mol $m^{-2}s^{-1}$ with an increase in $g_{s4}$ by 0.1 mol. After 2.5 h, however, the tendency was maintained only in 'Feliks'. In that time segment the reactions between $g_s$ *vs.* $P_N$ and $E$ got much weaker and between 7th and 155th min they showed very strong positive correlations, especially in 'Feliks' (Table 3).

## TRANSPIRATION AND NET PHOTOSYNTHETIC RATE RESPONSE TO LEAF PIERCING

At the first stage after piercing (up to 7th min) the leaves in 'Muller' reacted with a strong decrease in $P_{N1}$ (-35%) and $E_1$ (-22%) – (Tables 5, 6), which was, at the same time, correlated with an increase in $C_{i1}$ (Table 7). Then $P_{N2}$ was increasing in that cultivar in *log*-linear progression as a time function, giving two segments of the rate; up to 47th min by 0.055 µmol $m^{-2}$ $s^{-1}$ and from 48th to 220th

TABLE 3. Pearson's coefficients of correlation ($r$) and slopes ($b$) between $g_s$ *vs.* $C_i$, $E$ and $P_N$ parameters in time intervals for *cv.* 'Muller' and *cv.* 'Feliks' rape plants after leaf piercing.

| Interval [min] | Coefficient | $C_i$ | | $E$ | | $P_N$ | |
|---|---|---|---|---|---|---|---|
| | | 'Muller' | 'Feliks' | 'Muller' | 'Feliks' | 'Muller' | 'Feliks' |
| 1-6 | *r* | -0.81* | 0.86* | 0.99*** | 0.98*** | 0.91** | -0.28ns |
| | *b* | -49.6 | 2.27 | 0.90 | 0.77 | 0.85 | – |
| 7-23 | *r* | -0.90*** | -0.21ns | 0.99*** | 0.90*** | 0.97*** | 0.85*** |
| | *b* | -20.0 | – | 0.64 | 0.44 | 0.49 | 0.35 |
| 24-63 | *r* | -0.23ns | 0.90*** | 0.55*** | 0.99*** | 0.48** | 0.96*** |
| | *b* | – | 7.57 | 0.48 | 0.47 | 0.56 | 1.74 |
| 64-155 | *r* | 0.82*** | 0.91*** | 0.96*** | 0.99*** | 0.83*** | 0.96*** |
| | *b* | 10.2 | 7.78 | 0.45 | 0.33 | 0.17 | 1.85 |
| 156-220 | *r* | 0.21ns | 0.80*** | 0.24ns | 0.32** | 0.37** | 0.10ns |
| | *b* | – | 12.9 | – | 0.15 | 0.13 | – |
| 1-220 | *r* | 0.20* | 0.90*** | 0.95*** | 0.74*** | 0.86*** | 0.95*** |
| | *b* | 2.50 | 7.87 | 0.49 | 0.23 | 0.26 | 1.67 |

*r* – correlation coefficient by Pearson, *b* – linear coefficient of regression, *significance at $p$ = 0.05, ** $p$ = 0.01, *** $p$ = 0.001, ns – not significant

TABLE 4. Progress of intercellular $CO_2$ concentration [$C_i$] changes after leaf piercing of two rape cultivars according to the piecewise linear regressions of the time and with confident limits (CL).

| Break point of time [min] | 95% CL of time | $C_i$ [µmol mol$^{-1}$] | 95% CL of $C_i$ | Relative stepwise change [%] |
|---|---|---|---|---|
| *cv.* 'Muller' BBCH 30-31 | | | | |
| Starting = 0.0 | – | 211 | 194–229 | 100 |
| 3.0 | 0–3 | 258 | 255–262 | + 22 |
| 10.7 | 9–12 | 239 | 237–242 | - 7 |
| 46.3 | 44–49 | 226 | 224–227 | - 5 |
| 114 | 98–131 | 233 | 231–234 | + 3 |
| Ending = 220 | – | 238 | – | + 2 |
| *cv.* 'Feliks' BBCH 59 | | | | |
| Starting = 0.0 | – | 214 | 213–215 | 100 |
| 2.0 | 0–2 | 238 | 236–241 | + 11 |
| 8.8 | 8–9.6 | 226 | 225–227 | - 5 |
| 63.5 | 58.3–8.6 | 217 | 216–218 | - 4 |
| 146 | 137–155 | 226 | 225–227 | + 4 |
| Ending = 220 | – | 229 | – | + 1.1 |

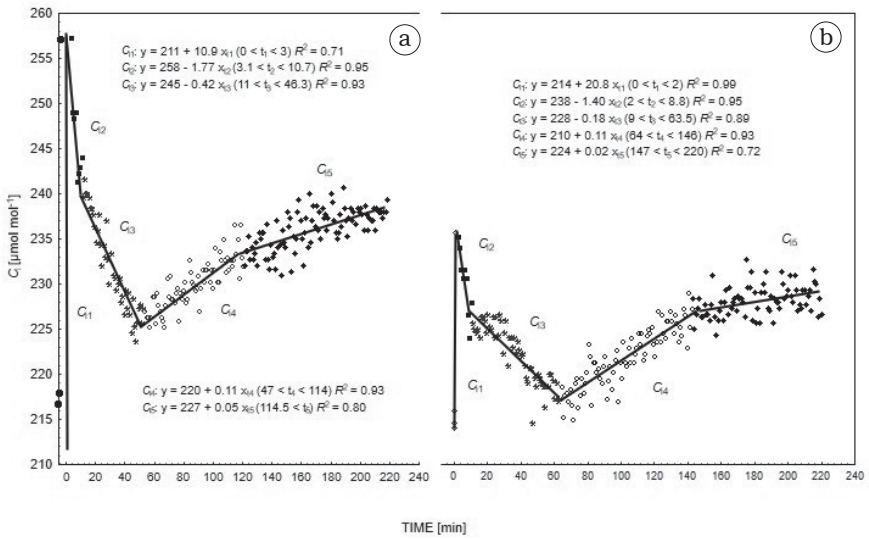


**Fig. 2**. Piecewise linear regressions for concentration of intercellular $CO_2$ after leaf piercing of rape plant. The confident limits (95% CL) of the linear parameters, the duration of each interval (t) and coefficients of determination ($R^2$); (**a**) *cv.* 'Muller', (**b**) *cv.* 'Felix'.

min by 0.006 µmol m$^{-2}$ s$^{-1}$ (Fig. 3a). The increase of $P_{N2}$ was accompanied by an increase in $C_{i4}$ only between 60$^{th}$ and 114$^{th}$ min; however, throughout the test the tendency was slightly negatively correlated ($r$ = -0.25) (Table 7). In 'Muller' there was found a very strong association of the increasing $E_{1\text{-}4}$ to increasing $P_{N2}$ up to 114$^{th}$ min of the test, after which both processes no longer showed a linear dependence. The very pattern of $E_3$ in 'Muller' was convergent with the $g_{s3}$ pattern, with the phase of 'dormancy' between 28$^{th}$ and 59$^{th}$ min, and two growth intervals, namely a rapid increase between 7$^{th}$ and 28$^{th}$ min by 0.017 mmol m$^{-2}$ s$^{-1}$ per min and 0.005 mmol m$^{-2}$ s$^{-1}$ slower over 60$^{th}$–114$^{th}$ min after piercing (Fig. 4a, Table 6). The $P_N$ and $E$ changes in 'Feliks' showed a completely differ-

TABLE 5. Progress of net photosynthetic rate [$P_N$] changes after leaf piercing of two rape cultivars according to the piecewise linear regressions of the time and with confident limits (CL).

| Break point of time [min] | $P_N$ [µmol m$^{-2}$ s$^{-1}$] | 95% CL of $P_N$ | Relative stepwise change [%] |
|---|---|---|---|
| *cv.* 'Muller' BBCH 30–31 | | | |
| Starting = 0.0 | 15.7 | – | 100 |
| 7.0 | 10.2 | 9.5–10.9 | - 35 |
| 47.0 | 12.7 | 12.1–13.3 | + 25 |
| Ending = 220 | 14.4 | 13.9–14.9 | + 13 |
| *cv.* 'Feliks' BBCH 59 | | | |
| Starting = 0.0 | 19.27 | 19.2–19.3 | 100 |
| 1.0 | 16.07 | 15.9–16.3 | - 17 |
| 25.5 | 17.35 | 17.1–17.6 | + 8 |
| 74.0 | 14.94 | 14.7–15.2 | - 14 |
| 148.0 | 17.83 | 17.6–18.1 | + 19 |
| Ending = 220 | 17.88 | – | + 0.3 |

TABLE 6. Progress of transpiration rate [$E$] changes after leaf piercing of two rape cultivars according to the piecewise linear regressions of the time and with confident limits (CL).

| Break point of time [min] | 95% CL of time | $E$ [mmol m$^{-2}$ s$^{-1}$] | 95% CL of $E$ | Relative stepwise change [%] |
|---|---|---|---|---|
| *cv.* 'Muller' BBCH 30–31 | | | | |
| Starting = 0.0 | – | 3.58 | – | 100 |
| 6.6 | 6.2–6.9 | 2.79 | 2.75–2.84 | - 22 |
| 27.5 | 27.3–28.1 | 3.15 | 3.14–3.16 | +13 |
| 59.5 | 55.4–63.5 | 3.17 | 3.17–3.17 | +0.6 |
| 114.0 | 110–118 | 3.48 | 3.46–3.50 | +10 |
| Ending = 220 | – | 3.56 | – | +2 |
| *cv.* 'Feliks' BBCH 59 | | | | |
| Starting = 0.0 | – | 4.05 | – | 100 |
| 9.0 | 6.5–10.6 | 3.82 | 3.79–3.88 | - 5.2 |
| 19.3 | 16.5–22.0 | 3.93 | 3.91–3.95 | + 2.9 |
| 65.5 | 47.5–83.4 | 3.34 | 3.11–3.57 | - 15 |
| 139.4 | 134–144 | 3.72 | 3.68–3.74 | + 11 |
| Ending = 220 | – | 3.63 | – | - 2.4 |

ent pattern after piercing. In the first minute after piercing there was recorded a decrease in $P_{N1}$ from 19.27 to 16.07 µmol m$^{-2}$ s$^{-1}$, i.e., by 17% (Table 5) and within 9 mins the decrease in the $E_1$ from 4.05 to 3.82 mmol m$^{-2}$ s$^{-1}$, i.e., by 5.2% (Table 6). Then, both processes started to increase; $P_{N2}$ for 24 mins at the rate of 0.05 µmol m$^{-2}$ s$^{-1}$ and $E_2$ for 10 mins at the rate of 0.009 mmol m$^{-2}$ s$^{-1}$ (Figs. 3b, 4b). At that time there was reported a significant negative dependence between a decrease in $P_{N1}$ and an increase in $C_{i1}$ (Table 7). At the third stage, which took place from 26$^{th}$ to 74$^{th}$ min there occurred a decrease in $P_{N3}$ by 0.04 µmol m$^{-2}$ s$^{-1}$ and $E_3$ by 0.013 mmol m$^{-2}$ s$^{-1}$. Directional convergence of those two processes was confirmed by the coefficient of correlation $r$ = 0.95. At the same time the

TABLE 7. Pearson's coefficients of correlation ($r$) and slopes ($b$) between $P_N$ *vs.* $C_i$ and $E$ parameters in time intervals for *cv.* 'Muller' and *cv.* 'Feliks' rape plants after leaf piercing.

| Coefficient | 'Muller' | | | 'Feliks' | | |
|---|---|---|---|---|---|---|
| | Interval [min] | $P_N$ *vs.* $C_i$ | $P_N$ *vs.* $E$ | Interval [min] | $P_N$ *vs.* $C_i$ | $P_N$ *vs.* $E$ |
| $r$ | 1–6 | -0.97*** | 0.93*** | 1–25 | -0.84*** | -0.1ns |
| $b$ | | -0.15 | 9.52 | | -0.11 | – |
| $r$ | 7–27 | -0.96*** | 0.97*** | 26–74 | 0.76** | 0.95** |
| $b$ | | -0.22 | 7.36 | | 0.16 | 3.65 |
| $r$ | 28–59 | -0.90*** | 0.50** | 75–148 | 0.75** | 0.95** |
| $b$ | | -0.11 | 7.46 | | 0.19 | 5.36 |
| $r$ | 60–114 | 0.46*** | 0.87*** | 149–220 | -0.37** | -0.19ns |
| $b$ | | 0.08 | 3.46 | | -0.05 | – |
| $r$ | 115–220 | -0.55*** | 0.00ns | 1–220 | 0.73*** | 0.65*** |
| $b$ | | -0.07 | – | | 0.15 | 3.64 |
| $r$ | 1–220 | -0.25** | 0.93*** | | | |
| $b$ | | -0.06 | 5.39 | | | |

$r$ – correlation coefficient by Pearson, $b$ – linear coefficient of regression, * significance at $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$, ns-not significant

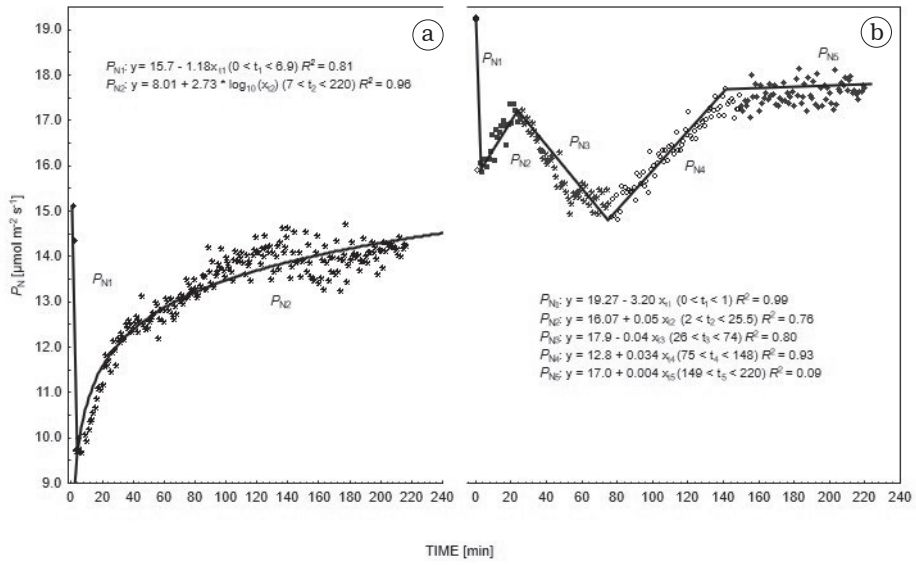


**Fig. 3**. Piecewise linear regressions and *log*-time regression for net photosynthetic rate after leaf piercing of rape plant, the duration of each interval (t) and coefficients of determination ($R^2$); (**a**) *cv.* 'Muller', (**b**) *cv.* 'Felix'.

dependence between $C_{i3}$ and $P_{N3}$ got reversed. At the successive stage there occurred rapid increasing in $P_{N4}$ (by 19%) (Table 5) and $E_4$ (by 11%) (Table 6), which lasted to 148$^{th}$ min and demonstrated strongly convergent processes. After that, to the end of the test, there was observed stabilization of both processes with non-significant correlation between them, an increase in $P_{N5}$ of 0.004 µmol m$^{-2}$ s$^{-1}$ and a slow decrease in $E_5$ by 0.0016 mmol m$^{-2}$ s$^{-1}$ (Figs. 3b, 4b). In stem leaves in 'Feliks' the pattern of $E$ was convergent in terms of the directions to the one observed for $P_N$. Those two processes were synchronized from 1$^{st}$ to 114$^{th}$ min of the test in plants at the rosette stage and from 26$^{th}$ to 148$^{th}$ min of

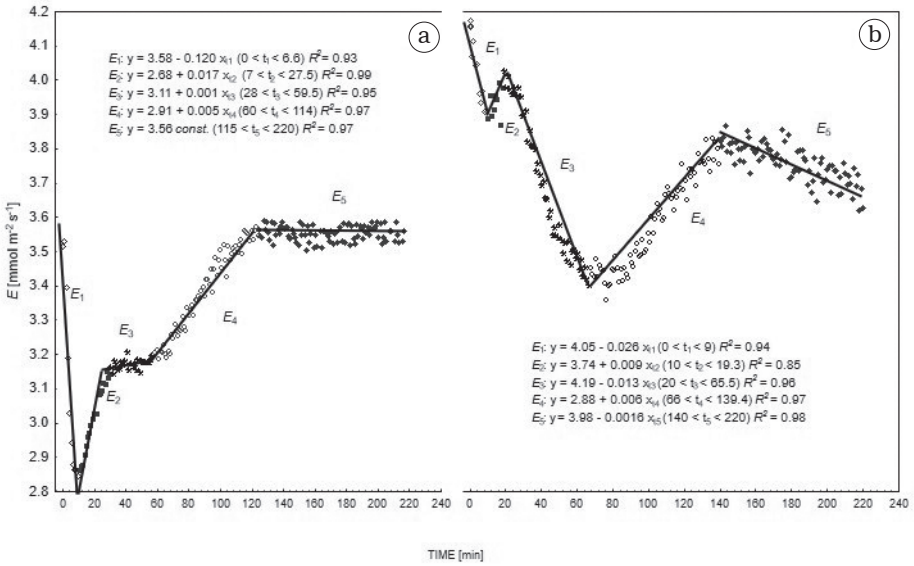


**Fig. 4**. Piecewise linear regressions for transpiration after leaf piercing of rape plant. The confident limits (95% CL) of the linear parameters, the duration of each interval (t) and coefficients of determination ($R^2$); (**a**) *cv.* 'Muller', (**b**) *cv.* 'Felix'.

the test for stem leaves (Table 7). Besides, throughout the time interval (1st–220th min) for 'Muller' the positive correlation ($r$) between $P_N$ and $E$ was very high 0.93 ($p < 0.001$) and for 'Feliks' – it was high $r = 0.65$ ($p < 0.001$).

## DISCUSSION

Gas exchange regulation is of great importance for water balance and the uptake of $CO_2$. Controlling $g_s$ is a complex process which depends on the water potential in leaves and on the intensity of transpiration as well as on such factors as the gradients of $CO_2$ concentration or the quality and intensity of light (Mott and Parkhurst, 1991; Sperry and Pockman, 1993; Messinger et al., 2006; Shimazaki et al., 2007).

Our research demonstrated that the plants of the same species, but at two BBCH stages, display different $g_s$ changes after leaf piercing. Blamowski et al. (2003) studied two species of the *Brassica* genus; radish and spring rape, both at the rosette stage, exposed to wounding: the oldest leaves removal or defoliation of the youngest ones with the apical stem. The oldest leaves removal resulted in the same reaction in both species; it increased the intensity of transpiration; however, it did not affect the intensity of assimilation. The defoliation of the youngest leaves with the apical meristem showed a completely different effect in radish and spring rape plants. In the case of radish, the authors do not relate any effect on the photosynthesis to the fact that at the rosette stage there occurs a very active acceptor of nutrient compounds (hypocotyl) which absorbs large amounts of carbon compounds supplied from leaves. This prevented the inhibition of photosynthesis, whereas in rape plants the defoliation of the youngest leaves with the apical meristem significantly decreased the intensity of assimilation, due to the accompanying increase of intercellular $CO_2$ concentration and slight fluctuations of the value of $g_s$, which pointed to non-stomatal limiting of photosynthesis. Koziołek et al. (2013) also described various stomatal conductance changes in leaves of shy plant (*Mimosa pudica*) due to abiotic factors, e.g., after thermal and light stimulation. The reaction after such stimulation on one part of the leaf showed a variable $g_s$ pattern for the neighboring leaf part in the system of open and rolled pinnules after touching. For the open leaf at the first $g_s$ stage it was a rapid growth about 40 s after thermal stimulation and taking about 1.7 mins, where $g_s$ reached the maximum value of 0.180 mol $m^{-2}s^{-1}$, after which it decreased to 0.050 mol $m^{-2}s^{-1}$ and reached the period of stabilization after about 33 mins, at the level slightly lower than the initial state. This process is very similar to that in 'Feliks' spring rape plant analyzed here after leaf piercing (Fig. 1b). Meanwhile, $g_s$ in closed leaflets of shy plant, due to thermal and light stress, changed similarly to the one described here for 'Muller' winter rape plants. At the initial value $g_s$ 0.150 mol $m^{-2}s^{-1}$ 8.3 min after induction there was a 3 fold decrease to 0.048 mol $m^{-2}s^{-1}$, then a slow increase started and after about 28.3 mins – $g_s$ stabilization which,

finally, achieved the value higher than before the stress (Koziołek et al., 2013). The mechanical stress due to cutting of the main vein in sunflower (*Heliathus annuus*) leaf, disturbed the processes of $P_N$, $E$ and stomatal conductance since it increased $g_s$ by 0.22–0.23 mol $m^{-2}s^{-1}$. The increase was compliant with the increase in $E$, which shows that the decrease in $P_N$ observed was not an effect of limiting the release of $CO_2$, which can occur upon decreased $g_s$ (Hanson et al., 2013). A decrease in $P_N$ and increase in $E$ were noted immediately after the cutting of the main vein. Photosynthesis reached the minimum value, on average, within 64 s after cutting-in, whereas the transpiration ratio assumed the highest value much later ($p$ = 0.0006), on average within 143 s after cutting-in (Henson et al., 2013). In our research, in pierced 'Muller' leaves $E$ first decreased by 22% and after 7 mins it started increasing rapidly to 27$^{th}$ min (+ 13%), then it became slower for 30 mins (+ 0.6%), and then increased by another 10% within 114 mins after piercing and it got stabilized to the initial value before the test. The plants of tomato *Lycopersicon esculentum* Mill. responded to chilling, as the stress factor, with stomatal conductance decreased by 32.2%, as compared with the control plants (Artuso et al., 2000). In reference to the stress-inducing factors of biotic origin, the reaction of $g_s$ in leaves is differently described by researchers. As reported by Nabity et al. (2013) for wild tobacco (*Nicotiana attenuata*) plants damaged by tobacco hornworm (goliath worm) (*Manduca sexta* L.) and Aldea et al. (2005) for soybean (*Glycine max* L., cv. Pioneer 93B15) plants damaged by Japanese beetles (*Popillia japonica*) and corn caterpillars (*Helicoverpa zea* Bodie), the stress induced by plant-eating insects did not have a significant effect on $g_s$ on wounded plants. Other biotic factors, e.g., viral infections, can cause changes in $g_s$, as demonstrated for mustard plants (*Brassica juncea* var. *tsatsai*) infected with turnip virus (Guo et al., 2005). No such reactions to mechanically-induced stress in other plants have been recorded. Nabity et al. (2013) found that the wild-type plants *Nicotiana attenuata* demonstrated a slight increase in $C_i$ (by 1%), while the modified plants – a 3.8% decrease due to *Manduca sexta* L. insects feeding. Guo et al. (2005), on the other hand, report on $C_i$ in leaves of the control plants and those infected with turnip mosaic virus in *Brassica juncea* var. *tsatsai*, being almost identical. Only Artuso et al. (2000) demonstrated that thermal stress (chilling) resulted in a decrease in the concentration of intercellular $CO_2$ in *Lycopersicon esculentum* Mill. (by 6.4%); however, the decrease was non-significant. According to Hinckley and Braatne (1994), stomatal conductance is inversely correlated with the concentration of carbon dioxide in leaves if tissues are adequately irrigated. Nardini et al. (2003) show linear dependence between gas exchange and water vapor conductance and the parameters are closely correlated with each other ($r^2$ = 0.987, $p < 0.01$). We found strong positive correlation between $g_s$ and $C_i$ ($r$ = 0.86) for stem leaf wounded ('Feliks'), while in 'Muller' – they negatively correlated with $r$ = -0.81, which means that the leaves at the rosette stage retained $CO_2$ during the decrease in $g_s$ over 1$^{st}$–23$^{rd}$ min, while the leaves at the flowering stage were losing intercellular $CO_2$ with a decrease in $g_s$ (from 1$^{st}$ to 6$^{th}$ min and from 24$^{th}$ to 63$^{rd}$ min). Photosynthesis and transpiration are, most frequently, correlated with each other due to the fact that stomata determine the conductance of water vapor and carbon dioxide (Farquhar and Sharkey, 1982). Mechanical leaf wounding in tomato resulted in an increase in the photosynthesis rate 1–5 mins after wounding (Herde et al., 1999). In our research, after the period of a rapid decrease, increasing $P_N$ took place from 2$^{nd}$ to 7$^{th}$ min, earlier in rosette leaves and later in stem leaves. However, the latter did not reach the stabilization of $P_N$ yet, showing successive decrease of $P_N$ by 14% after 25 mins and only after 75 mins $P_N$ started to get stabilized. Unlike 'Muller' plants at the rosette stage, there was demonstrated dependence of $P_N$ on time in *log*-linear progression: y ($P_N$) = 8.01 + 2.73 $\log_{10}$ (x $t_2$); 7 < $t_2$ < 220; $R^2$ = 0.96. The research reported by Hanson et al. (2013) shows that the response to the stress of cutting-in the midvein in sunflower was a fast decrease in $P_N$ by an average of 8.5 ± 4.1 μmol $CO_2$ $m^{-2}$ $s^{-1}$, i.e., by 40%, as compared with the status before the stress. Here, that decrease amounted to 35% at the rosette stage and to 31% in stem leaves, however, in the latter the decrease occurred in two-stages; 17% in the range from piercing to 1$^{st}$ min and 14% from 26$^{th}$ to 74$^{th}$ min. Midvein cutting-in in sunflower leaf also increased the process of $E$ by 1.3 ± 1.0 mmol $H_2O$ $m^{-2}$ $s^{-1}$ (Hanson et al., 2013). As reported by Artuso et al. (2000), processes $P_N$ and $E$ in tomato demonstrated a considerable decrease due to chilling stress by 37.7% and 29.5%, respectively, however, the authors do not describe how they were synchronized. Although genetic differences in photosynthetic capacity exist at intraspecific and interspecific levels, $P_N$ is considered as one of the potential, physiological, selection criteria for stress tolerance (Ashraf, 2004). The reactions of plants to the effect of biotic stresses, mostly triggered by insects or viral pathogens, visible through changes in $P_N$ and $E$ of leaves, were studied at many stages. Infecting mustard plants with turnip mosaic virus appeared an essential factor decreasing the $P_N$ by 52%, while for $E$ at the initial stage of infection it did not matter, after which there was observed an increase in $E$ and, at the final stage, a decrease, which was in no way related to the pattern of chang-

es in $P_N$ (Guo et al., 2005). Comparisons of the reaction of tobacco to two types of stress were investigated by Hlaváčková et al. (2002). The mechanical wounding of *Nicotiana benthamiana* leaf surface significantly decreased the process of $P_N$ measured after 11 days by 2 μmol $m^{-2}$ $s^{-1}$ and additional infection of those leaves with virus PPV (*plum pox potyvirus*) – by 4 μmol $m^{-2}$ $s^{-1}$, which, in relative values, accounted for 34% and 62%, as compared with the control (unwounded) leaves. Thirty-nine days after inoculation/mechanical wounding, a decrease in $P_N$ was, in both cases, similar and it accounted for 40%. For that reason the cited authors' claim that $P_N$ and $E$ are not synchronized after stress does not get confirmation; the authors state that at minimum $P_N$, $E$ was maximum. In our research those two processes were synchronized from 1st to 114th min of the test in plants at the rosette stage and from 26th to 148th minute of the test in plants at the stem stage. Besides, throughout all time intervals (1st–220th min) for 'Muller' the positive correlation ($r$) between $P_N$ and $E$ was very high 0.93 ($p < 0.001$) and for 'Feliks' – high 0.65 ($p < 0.001$).

We conclude that the only convergent process in both rape plant forms was observed for the concentration of intercellular $CO_2$. Here, after a rapid increase in $C_i$ (by 11–22%) taking 2-3 minutes after piercing, there occurred two stages of decrease and two stages of increase and in both leaf types the $C_i$ level at the end of the test exceeded the initial level by an average of 15–27 μmol $mol^{-1}$.

## AUTHORS' CONTRIBUTIONS

AW-P is the author of the concept, statistical analyses, wrote the manuscript, WK designed the research, collected data, wrote the manuscript, AN collected data, obtained funding, wrote the manuscript, MK collected data, MT-S prepared plants.

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