Regulation of cluster compactness and resistance to *Botrytis cinerea* with β-aminobutyric acid treatment in field-grown grapevine

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Summary

Our paper offers unique information regarding the effects of DL-β-aminobutyric acid (BABA) on grape cluster compactness and *Botrytis* bunch rot development. The impact of treatment was investigated on a native Hungarian grapevine cultivar, ‘Királyleányka’ (*Vitis vinifera* L.) during three seasons. The highly sensitive cultivar with thin skinned berries provided excellent samples for *Botrytis* bunch rot studies. Our objective was to study if BABA treatment contributes to decrease *Botrytis* infection by promoting looser clusters. For this purpose, the female sterility effect of BABA in grapevine flowers was also examined, which may result in looser clusters. Cluster compactness was characterized with two different indexes; bunch rot incidence was assessed in percentages. Ovaries of flowers were examined under epifluorescent microscopy. Significant differences were found in the compactness index (berry number/bunch length) between the control (2.87 ± 0.83 no./cm) and treated bunches in the case of 2.0 g L⁻¹ (2.18 ± 0.77 no./cm) and 3.0 g L⁻¹ (1.90 ± 0.72 no./cm) concentrations. Bunch rot incidence, however, was highly dependent on disease pressure influenced by the precipitation during ripening. In year 2013 all treatments gave significantly lower infection incidence, while the extremity of rain in 2012 and 2014, resulted in no epidemic or high infection clusters. For this purpose, the female sterility effect of BABA in grapevine flowers was also examined, which may result in looser clusters. Cluster compactness was characterized with two different indexes; bunch rot incidence was assessed in percentages. Ovaries of flowers were examined under epifluorescent microscopy. Significant differences were found in the compactness index (berry number/bunch length) between the control (2.87 ± 0.83 no./cm) and treated bunches in the case of 2.0 g L⁻¹ (2.18 ± 0.77 no./cm) and 3.0 g L⁻¹ (1.90 ± 0.72 no./cm) concentrations. Bunch rot incidence, however, was highly dependent on disease pressure influenced by the precipitation during ripening. In year 2013 all treatments gave significantly lower infection incidence, while the extremity of rain in 2012 and 2014, resulted in no epidemic or high infection, respectively. The treatment with 2.0 g L⁻¹ BABA concentration decreased cluster parameters and led to the lowest disease incidence. Microscopic studies proved that successful treatments on cluster structure can be traced back to the female sterility caused by BABA. Our results presented clear evidence for the effectiveness of BABA treatment on *Botrytis* bunch rot by promoting looser clusters.

Key words: BABA (DL-β-aminobutyric acid); *Botrytis* bunch rot; cluster compactness; epifluorescent microscopy; female sterility.

Introduction

*Botrytis* bunch rot of grape is a particular problem in the continental temperate regions of Europe with occasionally occurring wet macroclimate during bloom and berry ripening, that is favorable for disease development. However, several other variables play a direct or indirect role in development of the infection, e.g. susceptibility of the berries, cluster architecture, microclimate of the clusters (Vail and Marois 1991), canopy management (Werner et al. 2008), or plant nutrition (Keller et al. 2001, Cabanne and Donèche 2003, Valdes-Gomez et al. 2008). Keller et al. (2003) confirmed bloom as a critical developmental stage for infection, followed by latency until the berries begin to ripen. However, the correlation between the primary infection of flowers and the secondary infection of berries is not clear yet (Elmer and Michaelides 2004). Anatomical parameters of berries, like structure of epidermis, cuticle and wax content, play a more important role in cultivar resistance to bunch rot, than the antifungal host defence mechanisms (Gabler et al. 2003).

The above mentioned surface characters are influenced by the berry-to-berry contact, consequently by the cluster compactness which has high impact on *Botrytis* epidemics (Hed et al. 2009). There are several attempts, mainly in table grape cultivation, to find the proper chemical, that favourably influences cluster structure. A range of chemical components was evaluated in terms of effectiveness on cluster structure (Weaver and Pool 1971, Schuberg et al. 2011). Gibberellic acid treatment was successfully applied to loosen the clusters (Teszlák et al. 2005, Spies and Hill 2008). Foliar application of the non proteinogenic DL-β-aminobutyric acid (BABA) in table grape gave promising results in maintaining quality and controlling bunch rot infection under cold storage (El-Metwally et al. 2014). This chemical is an effective inducer of resistance against biotic and abiotic stresses (Jakab et al. 2001, 2005). Several studies showed, that BABA increased defense capability of the plants via fast hypersensitive response, callose deposition, lignin accumulation and PR protein synthesis (Hamiduzzaman et al. 2005, Tson et al. 2005, Cohen et al. 2011). It induced local and systemic resistance against downy mildew in grape leaves (Cohen et al. 1999). Besides the priming of pathogen-specific defense responses by BABA, it may have direct fungicidal effect as well. Treatment on field-grown grapevine controlled the downy mildew infection through inhibition of sporulation (Reuveni et al. 2001), and inhibited mycelial growth and germination of *Botrytis cinerea* on agar medium (Fischer et al. 2009). Furthermore, application of BABA during fertilization may reduce berry number through...
the induction of female sterility (Jakab et al. 2001). In the case of Arabidopsis flowers, BABA treatment as soil drench induced callose deposition in the micropylar region of the ovules, inhibiting the fertilization process (Kocsis and Jakab 2008). BABA application to flowering tomato plants resulted in a strong reduction in fruit set (Vaknin 2016), although as a root drench it did not repress seed development of tomato (Luna et al. 2016).

In our study, BABA treatment was tested to regulate cluster compactness and consequently to control bunch rot on field-grown grapevine. The study was carried out on one of the native cultivar in Hungary, ‘Királyleányka’ (synonyms: ‘Konigstochter’, ‘Feteasca regale’), whose clusters are highly susceptible to Botrytis infection. The cultivars indigenous in the Carpathian Basin are more susceptible to Botrytis bunch rot infection than the worldwide ones. Among them the studied cultivar is widely used for wine production in this region. Based on previous studies, we hypothesized that BABA may be capable of decreasing grapevine susceptibility to Botrytis infection through different ways, namely as a potent inducer of resistance, as direct fungicide (direct ways) and as inducer of female sterility (indirect way). According to our knowledge, the effects of BABA applied on grapevine flowers against bunch rot infection were analysed for the first time in this study.

Material and Methods

Experimental vineyard and maintenance: Field experiments were conducted in three consecutive seasons, from 2012 to 2014 on field-grown grapevines (Vitis vinifera L. ‘Királyleányka’) grafted onto Berl. x Rip. T. F. SO4. The experimental vineyard was located at the Institute for Viticulture and Oenology, Pécs, Hungary. The vineyard was planted in 1987, with vine-by-row spacing of 3.0 x 1.2 m in north-south orientation. All vines in this study were not sprayed especially for Botrytis bunch rot infection, although the following standard spray program was applied for control of other fungal diseases uniformly on whole experimental area: three applications of mancozeb (Dithane DG at 2.0 kg·ha⁻¹); two applications of folpet (Folpan 80 WDG at 1.25 kg·ha⁻¹); two applications of copper hydroxide (Copac Flow at 2.0 L·ha⁻¹); four applications of sulfur (Kumulus S at 2-4 kg·ha⁻¹); one application of proquinazid (Talendo at 0.25 L·ha⁻¹). Furthermore, in 2012 one application of tebuconazole + triadimenol + spiroxam (Falcon at 0.3 L·ha⁻¹); one application of metrafenon (Vivando at 0.25 L·ha⁻¹); in 2013, one application of meptil-dinokap (Karathane Star at 0.6 L·ha⁻¹); one application of miklobutanil (Talentum at 0.1 L·ha⁻¹) and in 2014, one application of metiram + ametoktradin (Enervin at 2.5 kg·ha⁻¹); two applications of meptil-dinokap (Karathane Star at 0.6 L·ha⁻¹); two applications of metrafenon (Vivando at 0.25 L·ha⁻¹); one application of tetraconazole + proquinazid (Talendo Extra at 0.25 L·ha⁻¹); one application of difenoconazole + cyflufenamid (Dynali at 0.5 L·ha⁻¹); one application of boskalid + kresoxim-metil (Collis SC at 0.4 L/ha).

Experimental performance: In the year 2012, treatments included 2.0 g·L⁻¹ BABA (DL-β-amino-n-butyrac acid, Sigma) concentration, while the next two years three concentrations (1.0 g·L⁻¹, 2.0 g·L⁻¹, 3.0 g·L⁻¹) were dissolved in water and sprayed on the flowers of ‘Királyleányka’ at BBCH 65 phenological stage (Lancashire et al. 1991). The applied concentrations of BABA treatment were based on our preliminary results on grey mould (Csikász-Krizsics et al. 2013) and Reunanen et al. (2001) field experiment in grapevine against Plasmopara viticola. Treatments were applied in the early morning (at 8-9 a.m.) before opening of the flowers. The bunches were collected and evaluated at harvest time (BBCH 89).

Data collection for the experiments: Treated and control clusters were removed and taken to the laboratory just before harvest. Compactness was assessed by the total number of berries per centimeter of the bunch (Pommer et al. 1996) in the year 2012. Bunch length included the length of the main rachis and that of the lateral branches. Rachis length was measured from the first lateral branch to the bottom of the cluster. In addition, the measured parameters were completed with the weight of the clusters and that of the berries in the year 2013. Thus, another compactness index derived by dividing bunch weight with the squared bunch length was calculated (Telio and Ibanez 2014). Bunch rot incidence was determined in percentage by visual assessment of the health status of the clusters (% of bunches infected) at harvest time.

Microscopic analysis of grapevine flowers: Inflorescences in the phenological stage BBCH 68 were harvested and stored in ethanol, glycerol and water (1:1:1 v/v) until use. The staining procedure was made according to Lu et al. (2011) with minor modifications. The flowers were fixed in 200 µL acetic acid and ethanol (3:1 v/v) for 1.5 h. To soften the tissues, they were submerged in 1 N NaOH for 15 min in 60 °C thermostat, then washed 3-times with distilled water and stained with 200 µL aniline blue (0.01 %) for 5-10 min. Callose depositions and callose-rich structures, e.g. pollen grains and pollen tubes fluoresced yellow under the epifluorescent microscope (Nicon Eclipse 80i microscope with UV light – adapted system, with illumination from an Osram HBO 100 W/2 mercury lamp). Micrographs were taken by the Spot Basic 4.0 software.

Statistical analyses: Data from three repetitions each with 10 clusters per treatments (n = 30) were used for evaluation of cluster compactness. Bunch rot incidence was calculated in percentages, based on 50 randomly selected clusters per treatments, visually observed as healthy or infected. For microscopic analysis 5-5 pollinated flowers per 4 clusters per treatments (80 flowers in all) from 2013 were used. Average values and standard deviation (SD) data were calculated using Microsoft Excel 2010 software. The significance of differences was assessed using Student’s t-test.

Results

Effects of BABA on cluster compactness and on ovary fertilization: The applied BABA concentration in 2012 was 2.0 g·L⁻¹. Control bunches were 21.4 ± 9.1 cm bearing 96.1 ± 36.6 berries, which resulted in 4.67 ± 1.39 cluster compactness index calculated as
Regulation of cluster compactness and resistance to Botrytis cinerea

The BABA treatment completed the expected effect in so far as the compactness index of treated bunches decreased by 15% (3.95 ± 1.12). Next year the concentration dependent efficiency of BABA treatments was investigated, when 1.0 g L⁻¹, 2.0 g L⁻¹ and 3.0 g L⁻¹ concentrations of BABA were applied (Table). Although the treatment with 1.0 g L⁻¹ did not result in significant differences in the measured parameters to the control, its compactness index decreased significantly these parameters except the bunch length. The weight of the berries and consequently that of the clusters were decreased also by the 3.0 g L⁻¹ concentration, compared to the control. Only this concentration affected bunch length, while berry number was not changed significantly. Cluster compactness of control bunches was significantly lower in the year 2013 than in the previous year because of the unfavorable fruit set. The treatments with 2.0 g L⁻¹ and 3.0 g L⁻¹ concentrations further loosened the clusters based on both indexes (Table, Fig. 1).

The treatments with 2.0 g L⁻¹ and 3.0 g L⁻¹ concentrations reduced significantly the weight of berries, while berry numbers were significantly lower only in the case of the 2.0 g L⁻¹ concentration. A post pollination study of the pistils of flowers treated by the 2.0 g L⁻¹ concentration offer an explanation for this phenomenon. Inflorescences were harvested in the phenological stage BBCH 68, in order to let prevail the female sterility induction of BABA (Fig. 2). In the case of control flowers, it was clearly observable that the pollen tube entered the ovules and there was no callose deposition at the micropylar region in 90% of the pollinated flowers studied (Fig. 2a). Based on our observations, in some flowers of the BABA-treated inflorescences (20%) pollen tubes did not reach the ovule, but they were interrupted about halfway in the ovary. Additionally, BABA induced callose deposition at the micropylar region and sometimes in the ovules body itself as well (Fig. 2b). Furthermore, there were BABA-treated flowers (about 30%), which presented both phenomena in the same ovary, namely there were fertilized and non-fertilized ovules, the latter with vigorous callose deposition at least at the micropylar region (Fig. 2c).

Botrytis bunch rot incidence: The cultivar 'Királyléányka' with medium sized, shouldered clusters and with thin skinned berries, provided excellent samples for Botrytis bunch rot studies (Fig. 1a). Infection incidence was 46.3% in 2013 on control clusters. In this year all BABA treatments gave significantly lower infection incidence compared to the control (Fig. 3). The most effective one proved to be the 2.0 g L⁻¹ concentration, which limited the 46% infection to less than 11.6%. The treatments with 1.0 g L⁻¹ and 3.0 g L⁻¹ concentrations resulted 17.1% and 33.0% infection incidences, respectively.

Table: Effect of BABA-treatment on different bunch parameters of 'Királyléányka' in 2013

<table>
<thead>
<tr>
<th>BABA (g·L⁻¹)</th>
<th>Measured parameters</th>
<th>Compactness index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW (g)</td>
<td>BL (cm)</td>
</tr>
<tr>
<td>1.0</td>
<td>85.8a ± 27.7</td>
<td>24.5a ± 4.8</td>
</tr>
<tr>
<td>2.0</td>
<td>60.3b ± 30.6</td>
<td>22.2a ± 6.5</td>
</tr>
<tr>
<td>3.0</td>
<td>63.4b ± 32.3</td>
<td>26.2b ± 7.4</td>
</tr>
<tr>
<td>Control</td>
<td>84.2a ± 26.5</td>
<td>21.5a ± 7.1</td>
</tr>
</tbody>
</table>

Values are means and standard deviations of data from 3 repetitions each with 10 clusters (n = 30). BW: Bunch weight; BL: Bunch length; NbB: Average number of berries per bunch; Wb: Weight of berries; no.: number. Means within a column followed by the same letter are not significantly different based on Student's t-test (p < 0.05).

Fig. 1: Control (a), 2.0 g L⁻¹ (b) and 3.0 g L⁻¹ (c) treated bunches of 'Királyléányka' in the year 2013. (Scale bar: 6 cm).
There was no bunch rot in the year 2012. The year was dry after veraison and during berry ripening, which means that the infection evaluated was mainly the result of primary infection of the flowers. Secondary infection of the clusters was improbable because of the following dry weather (August, September). The treatments of inflorescences in the year 2013, which significantly decreased Botrytis incidence, further highlight the significance of primary infection of the flowers. The last study year (2014) gave no significant differences regarding cluster compactness (data not shown), and provided the ineffectiveness of the treatments on Botrytis infection (Fig. 3). The unusually high amount of rain in most of the vegetation period in the year 2014 allowed high epidemics in spite of the treatments.

**Discussion**

The field-experiment showed that the application of BABA on grapevine inflorescences had positive effect on cluster compactness and reduced Botrytis infection. Although, disease pressure and weather conditions strongly influenced the results. Correlation between cluster character and bunch rot is well known, because plant growth regulators have long been applied on grapevines for induction of loose clusters and to restrict bunch rot attacks in fungicide-based management (Prior 2006, Zdunić et al. 2015). Among the many methods of quantifying grape cluster compactness in the literature, we used two different calculations based on three parameters, namely the berry number, bunch weight and bunch length (Tello and Ibáñez 2014). The indicator calculated as berry number per centimeter of a bunch was strongly correlated with bunch rot infection (Hed et al. 2009), while this metric had low contribution to cluster compactness in characterization of different cluster architecture of several cultivars and clones (Vail and Marois 1991). In our study, we calculated bunch length as the sum of the length of the main rachis and lateral branches, while Hed et al. (2009) measured only the rachis length and disregarded berries on the shoulder, because they rarely developed bunch rot in Vignoles’ grapes. In our case, calculation with the whole bunch length was justified by the bunch architecture of the cultivar, whose lateral branches bear several berries tending to bunch rot. The other compactness indicator we used was calculated with bunch weight and length, proposed by Tello and Ibáñez (2014). Regarding compactness indexes, the impact of BABA treatments should be reflected in decreased berry number and lower bunch weight, starting from the female sterility effect of BABA (Jakab et al. 2001).

Our microscopic studies of grapevine flowers revealed that the mechanisms behind the BABA-induced alteration of cluster architecture were associated with callose formation in the ovules and with inhibited pollen tube guidance in the ovary. These results are in agreement with previous studies that also established callose accumulation in grapevine leaves due to BABA treatment (Hamiduzzaman et al. 2005). Furthermore, inhibited fertilization through induction of callose deposition at the micropylar region and interrupted pollen tubes have also been observed in Arabidopsis flowers (Kocsis and Jakab 2008).
Bunch structure was modified significantly by different gibberellic acids and gibberellic acid inhibitors, while bunch rot incidence and severity showed no differences in the grapevine cultivar ‘Sauvignon blanc’ (MUNDY et al. 2014). There was no significant difference in the induction of loose clusters and Botrytis infection using a plant-growth regulator containing prohexadione-calcium (SCHILDBERGER et al. 2011). Our results confirm these observations, in case all concentrations decreased the disease incidence regardless of the effectiveness of treatments on bunch structure (see treatment with 1.0 g L⁻¹ concentration). This observation suggests additional mechanisms behind the observed decrease of Botrytis bunch rot.

In addition to the supposed effect of BABA treatment on cluster compactness, we trusted its further mechanisms leading to the reduction of Botrytis infection. BABA treated plants react faster and more effectively to biotic stress because of the priming of pathogen specific defence responses (SUNWOO et al. 1996, ZIMMELI et al. 2001, TON and MAUCH-MANI 2004). The protective effect of BABA can be based not only on induced resistance of the plant, but on direct toxicity of Botrytis cinerea, as it has been reported by FISCHER et al. (2009). Inhibition of sporulation, mycelial growth and germination has been observed (REUVENI et al. 2001, FISCHER et al. 2009). According to Csinkó-Krizesics et al.’s (2013) studies, although BABA can inhibit mycelial growth above 400 μg L⁻¹ concentration, its fungicidal effect was not observed. Evidence for BABA induced resistance in grapevine against fungal infections (FISCHER et al. 2009, SÁNEK et al. 2012) or against the Oomycete downy mildew (COHEN et al. 1999, REUVENI et al. 2001, HAMIDUZZAMAN et al. 2005, SLAUGHTER et al. 2008) has been described. The BABA-induced plant defence can explain that the treatment with 1.0 g L⁻¹ concentration of BABA which did not change the cluster compactness, could significantly reduce Botrytis bunch rot incidence. Our study gives explanation for looser clusters and decreased berry weight caused by BABA treatment, because this substance as inducer of female sterility inhibited fully or partly the fertilization of flowers.

However, a successful treatment cannot be guaranteed, because timing of treatments and meteorological conditions have decisive impact on the process. GIUDICE et al. (2004) emphasized the importance of timing of treatments with prohexadione-calcium, which decreased fruit set pre bloom and decreased berry weight post bloom. In our case, the treatments were justified at BBCH 65 phenological stage, when about fifty percent of the flower caps were fallen (LANCASHER et al. 1991). During this period BABA imposed its female sterility effect on those flowers which were not yet fertilized. Weather conditions around grape bloom also affect fruit set and abscission, which are in strong correlation with bunch rot infection (MOLITOR et al. 2016). In addition to the flowers, berries after veraison are also very susceptible to bunch rot, while young, immature berries are highly resistant to the disease (KILLIONEMI et al. 2015). In accordance with the model of GONZALEZ-DOMINGUEZ et al. (2015) our results highlight that primary infection of the inflorescences had significant effect on Botrytis bunch rot. However, secondary infection promoted by high humidity during ripening can further increase harvest lost by bunch rot.

Conclusion

Our paper shows original data on the effect of BABA treatment at full flowering on grape bunch architecture, flower fertility and Botrytis bunch rot. We demonstrated that BABA influenced cluster compactness through fully or partly inhibited fertilization in flowers, resulting in decreased number and weight of berries. Furthermore, the observation that all of the treatments decreased disease incidence regardless of bunch structure, emphasized the protective role of BABA through resistance induction or pathogen inhibition. This multiple effect prevailed in the case of 2.0 g L⁻¹ concentration, resulting decreased cluster parameters and also the lowest disease incidence. In field conditions, however, the outcome of the treatment depends on different environmental factors influencing secondary infection of the clusters.

Acknowledgement

This work was supported by the Hungarian Scientific Grant Agency (OTKA K101430). The present scientific contribution is dedicated to the 650th anniversary of the foundation of the University of Pécs, Hungary.

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Received August 16, 2017
Accepted December 19, 2017