Genetics and Plant Physiology – 2016, Volume 6(3–4), pp. 158–166

Available online at http://www.ifrg-bg.com

Research Article

EFFECT OF EXOGENOUS β-AMINO BUTYRIC ACID ON *EXO*- AND *ENDO*-CELLULAR pH AND REDOX STATE OF ASCORBATE IN LEAVES OF BARLEY SEEDLINGS (*HORDEUM VULGARE* L.)

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Received: *05 August 2016* Accepted: *14 November 2016*

Summary: An effect of exogenous β-amino butyric acid (BABA) on the pH of *exo*-cellular (apoplast) and *endo*-cellular (cytoplasm) compartments as well as on the redox state of most important component of plant antioxidants system, ascorbic acid (AA) in barley seedlings (*Hordeum vulgare* L.) has been investigated. It was found that treatment of freshly cut leaves with BABA (10^{-3}M) caused immediate though reversible alterations in pH balance between the respective fluids. Thus, pH of the apoplast became basified by ≈ 0.3 pH units after the first ≈ 20 min of incubation, while the cytoplasm was acidified leading to decrease of its pH value by 0.6 units after 44 min. The pH changes were accompanied by a decrease (4.9 times) in redox state of ascorbate because of an increase in its oxidized form, the dehydroascorbic acid (DHA), and leaving the overall ascorbate amount (AA+DHA) roughly intact within 60 min after starting the incubation with BABA. The apoplastic and cytoplasmic pH changes are considered as primary event that altered ascorbate redox status (AA/DHA) without affecting AA synthesis in the beginning of BABA (10^{-3}M) action.

Keywords: Barley; green seedlings; BABA; pH apoplast − cytoplasm; ascorbate redox state; fluorescent pH indicators.

Abbreviations: AA – ascorbic acid; BABA – β-amino butyric acid, DHA – dehydroascorbic acid; FITC-D – fluorescein isothiocyanate–dextrane; ROS – reactive oxygen species.

Citation: Savchenko G. E., Kabashnikova L. F. , Abramchik L. M., 2016. Effect of exogenous β-amino butyric acid on *exo*- and *endo*-cellular pH and redox state of ascorbate in leaves of barley seedlings (*Hordeum vulgare* L.). *Genetics and Plant Physiology*, 6(3–4): 158–166.

INTRODUCTION

Increase of a general unspecific resistance of plants against adversities of both biotic and abiotic origin provided by induction of natural defensive mechanisms is one of the core problems in the contemporary horticulture. During the last two decades, priming that actually represents anything but sensibilization of the immune system of a plant against a pathogen with a low molecular organic compound has established itself as an efficient tool for a complex plant

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protection (Beckers and Conrath, 2007). One of the efficient agents of that kind is proved to be β-amino butyric acid (BABA) (Cohen, 1993). Detailed analysis of the consequences of BABA action in certain instances reveals however that expected priming effect of BABA is rather overwhelmed by an undesired growth inhibition thus reflecting its stressoric activity which becomes particularly evident in healthy plants (Schwarzenbacher et al., 2014). Hence, necessary prerequisite for development of BABA based plant protection agents requires a careful adjustment of its effective concentration and detailed determination of its activity profile towards a particular plant. Special interest in this respect represent methods for express evaluation of the possible adverse effects of BABA.

An important progress towards elucidation of the complex mechanisms of inducing action of BABA had been recently made by Luna et al. (2014), who identified a receptor responsible for a binding the exogenic BABA. To this end, we were particularly interested in events after the treatment of barley leaves with BABA that might lead to an alteration of the important regulatory parameters triggered by the binding of BABA to the receptor. Among the variety of the possible scenarios, one of the first responses to begin with might be the change of pH in different cell compartments, which could play regulatory role in many cell processes. Thus, in the apoplast, where all the primary sensors of the stress signals are localized, pH changes may influence decoding mechanisms as well as signal transmission to the regulatory proteins (Clarkson, 2007). Consequently, the transporting function of the apoplast defines to significant extent an internal media of the cytosol, whose pH changes would in turn trigger rapid mechanisms influencing the activity of membrane and ion transporting systems. pH changes taking place in different cell compartments also reflect deviations in other ionic concentrations and hence detection of pH becomes a particularly important facet in monitoring of stress effects as well as in a study of induced resistance mechanisms. On the other hand, the activity level of cell oxidative metabolism defines stationary pH value which in turn influences the activity of a manifold of enzymes, particularly those responsible for the regulation of the level of active forms of oxygen (ROS). Detoxication of the latter is effectively rendered by ascorbate, the most important component of plant antioxidant system. In this work we investigated the changes in *exo*- and *endo*-cellular pH and redox state of ascorbate in barley seedlings as important indicators of rapid response caused by exogenic BABA.

MATERIALS AND METODS

Plant materials and growth conditions

As an object for this study, green barley seedlings of cultivar "Magutny" were used. The seedlings were grown under laboratory conditions using a tap water at 23°C and under irradiation with polychromatic white light (intensity: 120 $\mu mol.m^2.s^{-1}$) over 14 h periods per day. Primary leaves of 7-day-old seedlings were used in these experiments. The seedlings were about 10 cm tall, and the secondary leaf was not formed yet.

For pH monitoring, first the fluorescent probes were inserted into detached leaves. Then, 1 mM aqueous solution of BABA

(Sigma) was introduced into the leaf through the cut end. This is the highest BABA concentration that was applied to barley plant (Walters et al., 2014).

Immediately before fluorescence measuring, 1 cm of leaf top was removed, and remaining upper part was used for the measurement. The size of the leaf sample was taken to match the fluorimeter's measuring chamber (6×8 mm).

For ascorbate analysis, 1 mM BABA solution was injected into detached primary leaves through the cut end within 60 min. The control leaves were treated with distilled water. The upper part (2 cm) of the leaf reduced by 1 cm from the top was analyzed.

pH measurements of apoplast and cytoplasm

pH monitoring of the apoplast was performed *in vivo* using as a probe pH sensitive fluorescein isothiocyanate covalently bind to polydextrane (FITC-D) of molecular weight 4kDa. The pH measurements in apoplast based on double excitation technique (Hoffman and Kosegarten, 1995) were problematic due to quite narrow gap between peaks corresponding to ν_{max} for absorption and emission (493 and 520 nm respectively) in the fluorescence spectra of FITC-D (imperfection in the resolution of available spectrometer SOLAR 2203, Minsk, Belarus). Therefore, we were forced to excite FITC fluorescence with the light of higher frequency (465 nm) than it would be required to match its absorption maximum (vide infra). Plausibility of such a shift was justified by the fact that in a physiologically significant pH range (e. g., 5.0 – 7.0) FITC occurs as a mixture of neutral and anionic form, displaying relatively broad absorption spectra in the range between 450 and 470 nm (Sjoback et al., 1995). Furthermore, there are two main peaks (at 520 and 550-555 nm) in the emission spectra of aqueous solution of FITC-D (at 465 nm excitation), and their fluorescence intensities change differently depending on pH (Savchenko et al., 2014). Therefore, the ratio of fluorescence intensity between peaks at 520 and 555 nm, namely $I_{520(ex465)}/I_{555(ex465)}$, could be used as an internal standard allowing to enhance the accuracy of pH measurements. To calibrate dependency of $I_{520(ex465)}/I_{555(ex465)}$ on pH, FITC-D (the final concentration 10^{-3} M) was mixed with the 20 mM MES buffers of the desired pH. In physiologically important pH range the dependence of this ratio on pH value, could be approximated as linear one (Fig. 1).

For pH measurement in the apoplast, the cut leaf area was immersed in aqueous solution of FITC-D (1 mM) over at least 3 h to allow the probe (FITC-D) to penetrate into the apoplast and to be transported to leaf upper compartments by transpiration pull.

For monitoring of the pH changes caused by BABA, the pre-loaded with fluorescent probe leaves were immersed with their cut side into aqueous BABA solution, hold there for a fixed time intervals, and then the fluorescence spectra were recorded. The pH changes in the cytoplasm were estimated using pH sensitive probe pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid, Sigma) (Giuliano and Gilles, 1987). The probe (10^{-3}M) was allowed to penetrate inside the leaf by the transpiration pull. Leaf cut edge was immersed into aqueous probe solution over 1 h. Then, the leaf

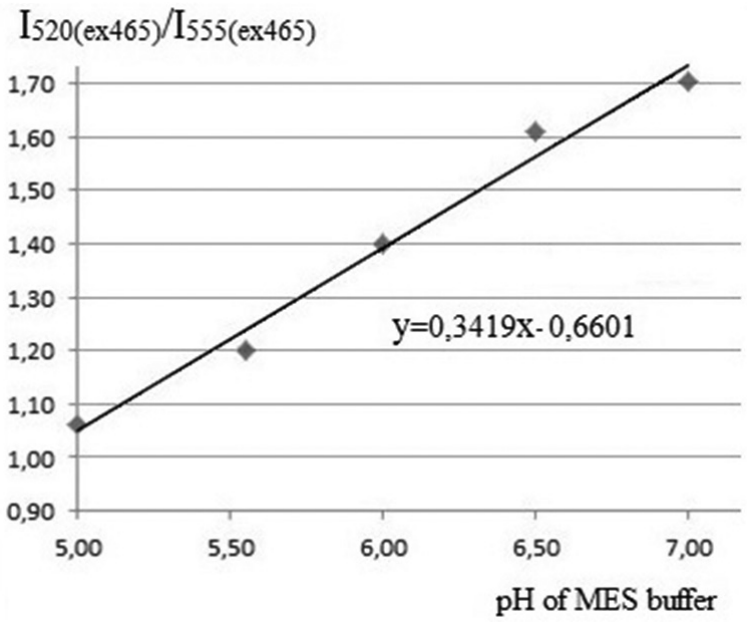

Figure 1. The linear section of the dependence of $I_{520(ex465)}/I_{555(ex465)}$ ratio for FITC-D (10^{-3}M) on pH (MES buffer); ex465 nm − the exciting light. $I_{520\ (ex.465)}/I_{555\ (ex.465)}$ is fluorescence intensity ratio of I_{520} at exciting light 465 nm to I_{555} at exciting light 465 nm.

was transferred into distilled water and left there over 1 h to remove the residual probe from the intercellular compartments (Oja et al., 1999). After that the leaf was immersed into aqueous solution of BABA, hold there for a fixed time intervals, and fluorescence spectra were recorded.

An array of fluorescence spectra was obtained where every time interval of exposition in BABA solution corresponded to separate leaves treated under the standard conditions.

Noteworthy, pyranine has two absorption maximums (at 404 and 456 nm) and one (at 520 nm) in the emission fluorescence spectrum. Intensity of fluorescence at 520 nm depends on wavelength of the exciting light as well as on pH value (Giuliano and Gilles, 1987). So, the fluorescence must be recorded at 520 nm, but excited at 404 and 456 nm (double excitation technique).

A linear section of the dependence of fluorescence intensity ratio of I_{520} at exciting light 404 nm to I_{520} at exciting light 456 nm, namely $I_{520\ (ex.404}/I_{520\ (ex.456)}$, on pH value is shown on Fig. 2.

All fluorescence measurements were conducted using fluorimetric chamber for solid samples.

For calibration, a cuvette (1 mm thick) was used containing solution of a probe, or a glass plate with leaves (both turned under an angle of 30 degrees towards the incoming light).

After exposure to fluorescent probes and BABA, the leaves were placed between the glass plates in a single-skin manner and orientated in the way that enables the analysis of similar leaf area.

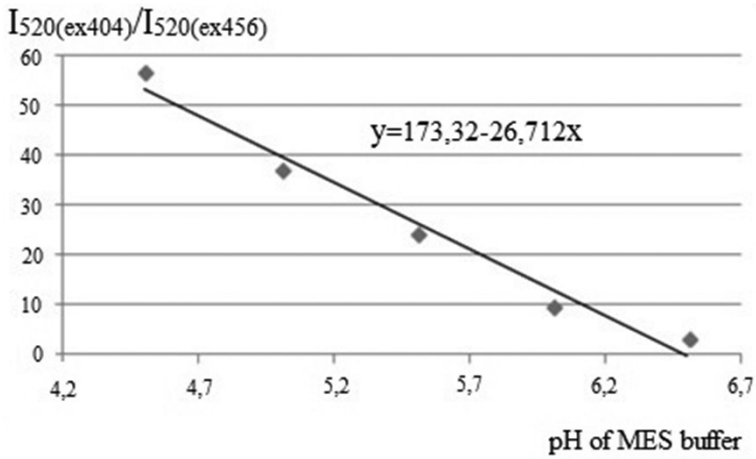

Figure 2. The linear section of the dependence of pyranine (10^{-3}M) fluorescence intensity at 520 nm on pH (MES buffer); the fluorescence intensity was recorded at two wavelengths of exciting light (404 and 456 nm); $I_{520\ (ex.404)}/I_{520\ (ex.456)}$ is fluorescence intensity ratio of I_{520} at exciting light 404 nm to I_{520} at exciting light 456 nm.

To accelerate registration, up to 5 cuttings were placed on the glass. The size of the fluorimeter registration window allowed simultaneous analysis of two cuttings per leaf. Therefore, by changing the position of glasses after each measurement, it was possible to analyze three leaf biologic samples.

Determination of the ascorbate content and its redox state

Ascorbate concentration was measured according to modified Okamura's method. The method is based on spectrophotometric determination of Fe(II)[bipy]$_2$ forming as a result of oxidation of ascorbic acid (AA) with Fe(III) and absorbing at 524 nm (Law et al., 1983).

Frozen in liquid nitrogen leaf 2-cm-cuttings (typically, 500 mg fresh weight) were quickly crushed in chilled mortar and triturated with aqueous solution of sulfosalicylic acid. Then, the resulting suspension was centrifuged and the supernatant solution was neutralized with alkaline solution to pH = 5.5–6.5. Aliquots of this solution were used for determination of the overall (AA+DHA) and reduced ascorbate (AA) content in the leaf tissue.

The procedure is suited primarily for measurement of the reduced form of ascorbate (AA), whereas the plant tissue may contain ascorbate in both reduced (AA) and oxidized (DHA) form, simultaneously. To determine the content of residual DHA in the sample, an aliquot corresponding to the sample with content of AA already known from the parallel experiment, was reduced to AA with an excess of dithiothreitol. Then, the overall ascorbate content was determined as described above for the partial one for AA, and DHA content was thus obtained as a difference between overall ascorbate (AA+DHA) and the reduced ascorbate (AA) (Dremuk and Shalygo, 2012).

Redox state of ascorbate was determined as a ratio of its reduced and oxidized forms (AA/DHA) or (AA/AA+DHA).

In statistical data processing the table (*t)* values depending on significance level (*P*) were used (Rokitskii, 1973).

RESULTS AND DISCUSSION

Changes of pH values in the apoplast of samples (cuttings) from the upper part of primary barley leaves upon treatment with BABA are shown on Fig. 3. Thus, 7 minutes after starting the incubation in BABA solution, pH in the apoplast was measured as ≈ 5.1 (5.05±0.09); it reached the value of 5.34±0.10 at 19 min and decreased to 5.18 at 27 min. P(t) between pH 5.05 (n_1=4) and 5.34 (n_2=7) < 0.1 > 0.05, t=1,93.

Figure 4 demonstrates BABA-induced changes in cytoplasmic pH; the pH response was recorded by the fluorescence of the pH indicator pyranine [the ratio $I_{520\ (ex.404)}/I_{520\ (ex.456)}$] pre-installed in the cytoplasm.

Interestingly, an acidification of the cytoplasm was observed even in control leaves incubated in distilled water (Fig. 4, ■), probably, due to traumatic stress. However, the acidification of the cytoplasm in the leaves incubated with BABA (Fig. 4, ♦) exceeded that in the control ones. The pH value in the examined part of the leaf changed from 6.0 to 5.4 (ΔpH = 0.6) within the initial incubation time of 44 min. The $I_{520\ (ex.404)}/I_{520\ (ex.456)}$ ratio was calculated as 12.5±0.9 (pH=6.0, n=4) for control, and 30.0±2.3 (pH=5.4, n=4) for the treatment; the differences between both variants were statistically reliable: t=7.09, P(t)<0.001. After ≈ 50 min of incubation with BABA a basification of

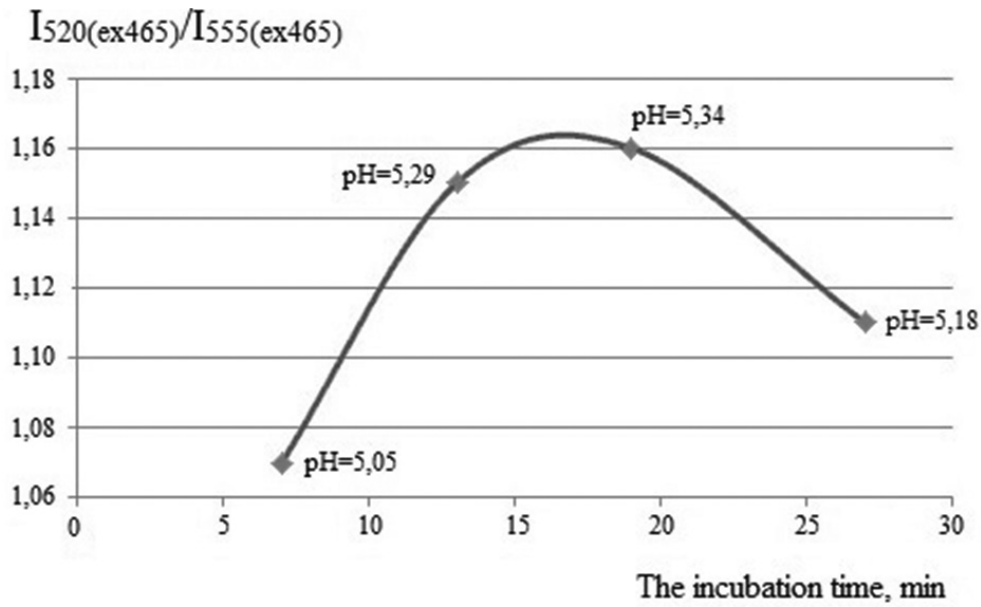

Figure 3. BABA-induced pH responses of barley leaf apoplast. The pH changes were expressed by means of fluorescence intensity ratio $I_{520(ex465)}/I_{555(ex465)}$ for FITC-D. The ratio is presented as a function of the incubation time of the cut leaves in BABA. pH values were calculated using the calibration curve (Fig.1).

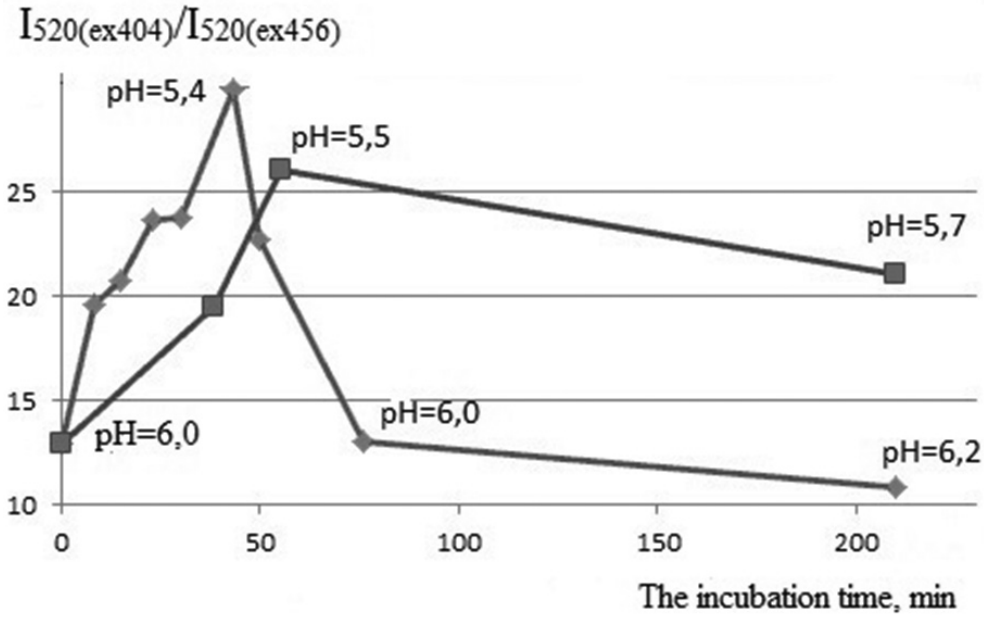

Figure 4. BABA-induced pH responses of barley leaf cytoplasm. The pH changes were examined by fluorescence intensity ratio [$I_{520\ (ex.404)}$)/$I_{520\ (ex.456)}$] for pyranine. The ratio is presented as a function of the incubation time of cut barley leaves in BABA (♦) or water (■). pH values were calculated using the calibration curve (Fig. 2).

the cytoplasm was observed; after 3.5 h pH values of treated and control leaves were measured as 6.2 and 5.7 (ΔpH = 0.5), respectively. The $I_{520\ (ex.404}$)/$I_{520\ (ex.456)}$ ratio was 10.81±0.75 (pH=6.2, n=4) for treated leaves, and 21.11±0.96 (pH=5.7, n=4) for control ones; respectively the differences between them were statistically reliable (t=8.44, P(t)<0.001).

So, we demonstrate pH fluctuations in both, apoplast and cytoplasm of cut barley leaf upon incubation in BABA solution. The basification of the apoplast (ΔpH=0.29) within the first ≈20 min was probably attributed to the reduced activity of proton pumps, however other factors could be considered as well. The data of fluorescent probing clearly indicate that BABA plays a role in the regulation of intracellular pH. However, the underlying mechanism of this obviously long-range signal is not fully understood, and the available data are rather inconsistent. Indeed, according to the radiolabeling experiments, the exogenous BABA was associated with the cell wall and cannot enter the protoplast (Cohen, 1994), however a presence of BABA receptor in endoplasmic reticulum membranes was suggested recently (Luna et al., 2014).

Table 1 summarizes the data on the status and redox state of the major plant antioxidant, ascorbic acid (AA), upon incubation of cut barley leaves in BABA solution. Total ascorbate content (AA+DHA) did not change within 60 min after the beginning of incubation; however the content of oxidized derivative, dehydroascorbic acid (DHA), increased under the action of BABA. Respectively,

Table 1. Content of ascorbate forms (μmol g^{-1} fresh weight) in cuttings (2 cm in length) from the upper part of primary barley leaves incubated for 60 min in distilled water (control), or in BABA (1 mM) solution.

Treatment	Total ascorbate (AA+DHA)	Ascorbic acid (AA)	Dehydroascorbic acid (DHA)	AA/DHA
Water (control)	2.69±0.09	2.19±0.001	0.50	4.38
BABA (1 mM)	2.59±0.06	1.23±0.006	1.36	0.90

AA/DHA ratio was 4.38 for control, and 0.90 for BABA treatment (the difference is 4.9 times). It is known that the main portion of ascorbate is negatively charged at physiological pH, and its diffusion through the lipid layer is not possible while uncharged DHA is able to pass the membrane. Besides, the apoplast lacks the enzyme able to reduce DHA (Gallie, 2013). Therefore, increased content of DHA in the presence of BABA allows to suppose that ascorbate is delivered in the apoplast as its DHA precursor. As the size of both, simplast and apoplast AA pool, as well as its redox state is regulated by the cytosolic DHA reductase (Heber et al., 2003), it could be supposed that DHA reductase activity is changed under the action of exogenous BABA. Obviously, the fluctuations in pH of the apoplast and the cytoplasm of barley leaves, were caused by exogenous BABA that is pulled in the apoplast by the transpiration stream. These fluctuations influenced the redox status of ascorbate without affecting its synthesis. Therefore, apoplastic and cytoplasmic pH changes could be considered as a primary event that altered AA/DHA redox status. It is quite possible that an assessment of the redox state of ascorbate over a wide range of BABA concentrations could provide an option to distinguish between stress-inducing and immunomodulatory activities of BABA.

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