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RESEARCH ARTICLE

Histochemical Examination of Invertase Activities in the Growing Tip of the Plant Root

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Abstract:

Background:

A growing part of the root is one of the most active sinks for sucrose coming from source leaves through the phloem. In the root, sucrose is unloaded from conducting bundles and is distributed among the surrounding cells. To be involved in the metabolism, sucrose should disintegrate into hexoses by means of degrading enzymes.

Aims:

The aim of this research was to explore the possibility of the involvement of one such enzymes, invertase, in phloem unloading as well as distribution of its activity in the functionally different tissues of the plant root tips.

Method:

To estimate the enzyme activities in root tissues, we applied two techniques: the histochemical method using nitro blue tetrazolium. The localization of phloem unloading was studied with carboxyfluorescein, a fluorescent marker for symplastic transport.

Results:

Invertase activity was not detected in the apical part of the meristem. It appeared only between the basal part of this zone and the beginning of the elongation zone. There is the root phloem unloading in that area. Invertase activity increased with increasing the distance from the root tip and reached the highest values in the region of cell transition to elongation and in the elongation zone. The activities of the enzyme varied in different tissues of the same zone and sometimes in the neighboring cells of the same tissue. Biochemical determination of invertase activity was made in the maize root segments coincident to the zones of meristem, cell elongation and differentiation. The results of both methods of determination of invertase activity were in agreement.

Conclusion:

It was concluded that phloem unloading correlated with invertase activity, possibly because of the activation of invertase by unloaded sucrose. Invertase is one of the factors involved in the processes preparing the cells for their transition to elongation because the concentration of osmotically active hexoses increases after cleavage of sucrose, that stimulates water entry into the cells, which is necessary for elongation growth.

Keywords: *Zea mays* L., *Cucumis melo* L., Root tip, Invertase, Phloem unloading, Histochemistry.

1. INTRODUCTION

A growing root tip is one of the most active sinks for organic compounds in plants. Growth processes which take place in this part of the root demand a great amount of substrates for the diverse syntheses and for the respiration. The rate of growth of the root tips depends on the supply of photoassimilates which move towards the roots from the donor

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leaves *via* the phloem. The main components of the photoassimilates are molecules of sucrose and/or oligosaccharides (raffinose, stahyose, verbascose). In the roots, sugars unloaded from the phloem are distributed among sink cells to supply them the energy (ATP) and carbon [1, 2]. Efflux from the sieve element/companion cells complex may be either symplastic through interconnecting plasmodesmata, or apoplastic into the surrounding cell wall matrix [1, 3 - 5]. Sucrose is a metabolically inert molecule and could participate in metabolism only after being cleaved into hexoses by invertase (EC 3.2.1.26) or sucrose synthase (EC 2.4.1.13). Therefore, activity of these enzymes could limit the rate of metabolism.On the contrary, the rate of metabolization of imported assimilates determines the sink capacity of the tissues and thus assimilate inflow and phloem unloading [6, 7].

Invertase and sucrose synthase have different effects on the osmotic potential of the cell. If sucrose synthase is active, then the degradation of sucrose leads to form one fructose molecule. Second breakdown product, UDPG, is actively involved in the metabolism and does not accumulate in significant quantities. Products of sucrose hydrolysis by invertase are molecules of two hexoses, glucose and fructose. As a result, the osmotic pressure in the cells is doubled which promotes active growth [6, 7]. The products of sucrose hydrolysis by invertase are mainly used in glycolysis; they are the most active in growing organs [6 - 8]. Since invertase is highly active in creating osmotic potential and in maintaining the metabolic activity of cells, therefore in the maintenance of growth, it primarily attracted our attention.

Distribution of invertase activities has been studied in more details in storage organs, which are especially practically important. Less attention was paid to "utilizing" sinks, among them, the growing root tip is the least studied [9]. Meanwhile, in the root tip, researcher can easily discern the zone in which the cells are predominant in various stages of development – meristem, elongation and differentiation zones. Activities of invertase in these zones have been earlier studied in the homogenates after cutting of the root into individual segments [10 - 12]. Already in these studies, the main features of the distribution of invertase on the primary root were found. As expected, the greatest activity was in the zone of intensive elongating cells. The activity failed as the cells matured. But such studies cannot accurately delineate the boundaries of the zones and coincidence of these segments with the root growth zones could be determined only roughly. It was also impossible to characterize individual cells within a particular root zone. Invertase activity remained unexplored in the transition region from one zone to another, which is the most interesting for understanding the interrelations between enzyme activities and tissue physiological functions.

More accurate data can be obtained by immunochemical methods, but they can determine the localization of the enzyme protein, but not its activity. We applied a histochemical method to estimate the enzyme activity in different zones, tissues, and cells of the root tip. This method has been earlier applied for root investigations [13, 14]; however, the cells of different zones and tissues were not characterized well. In this study, we would like to fill this gap. The objective of this work was histochemically determining invertase activity in the cells of different root zones using seedlings of maize, wheat and melon. Distribution of the invertase activity was compared to the localization of phloem unloading, to find out the relationship between these processes. Special attention was paid to study the enzyme activity during the transition of meristematic cells to elongation. The histochemical method also permitted us to compare the enzyme activity in functionally different tissues within a single maize root zone: rhizodermis, cortex, endodermis, pericycle, and in stele. Results of histochemical analysis were compared with the data of the biochemical determination of invertase activity in the segments of root tip of maize seedlings.

2. MATERIALS AND METHODS

2.1. Plant Material

The main object of the study was 3-day-old seedlings of maize (*Zea mays* L., cv. Odesskaya 10). In some experiments, we used winter wheat seedlings (*Triticum aestivum* L., cv. Mironovskaya 808) and melon (*Cucumis melo* L., cv. Serpyanka) seedlings. Seedlings were grown in darkness on filter paper moisted by tap water in a growth chamber at 27°C.

2.2. Histochemical Determination of Invertase Activity

We used the technique which was described in the work of Sergeeva and Vreugdenhil [13]. Longitudinal sections of maize root tips 120-300 μm thick were obtained with a sledge microtome. Transverse sections were cut free hand with razor blade. The sections were fixed immediately in the fixation mixture (2% paraformaldehyde, 2% polyvinylpyrrolidone 40, and 0.001 M dithiothreitol, pH 7.0). In some experiments, the entire root tips were fixed in the same fixation mixture. Fixation was performed for 1 h at 4°C. The sections and the root tips were washed with distilled

water 3 times for 1 h each and then overnight at 4°C.

To detect the enzyme activity, sections or root tips were incubated in the incubation medium for 1 hour at 30°C. The incubation medium contained 38 mM potassium-phosphate buffer, pH 6.0, 25 U of glucose oxidase, 0.024% of nitro blue tetrazolium, and 0.014% of phenasine metasulfate. The reaction was started by adding sucrose at a final concentration of 29 mM and was stopped by washing sections or root tips in distilled water. The reaction led to the reduction of nitro blue tetrazolium with the appearance of blue product. Enzyme activity was assessed from the presence of blue color. Control sections or root tips were incubated in the absence of sucrose, and in this case, any reaction did not get started and the dye remained colorless.

Sections were photographed with a JVC TK-C1480E camera (768 x 576 pixels) fastened on the Amplival microscope (Carl Zeiss, Germany) and attached to the computer. The process was controlled by the computer program Mecos.

2.3. Localization of Phloem Unloading Zone

For this purpose, the fine roots of melon seedlings were used. In this case, the color identification is not required cutting slices. The zone of phloem unloading was determined from the appearance of fluorescent marker, carboxyfluorescein (CF) in the seedling roots after the application of 10 µl of its diacetate ester (CFDA) on cotyledons [4, 15]. CFDA (Sigma, USA) was dissolved in dimethylsulfoxide to a concentration of 43.5 mM. This stock solution was diluted with distilled water with the concentration of 217 µM, pH 5.5. Localization of fluorescent label in the roots was detected using a Univar fluorescent microscope (Reichert Jung, Austria); the excitation wavelength was 480 nm and the emission wavelength was 515 nm.

We used 10 seedlings of each species to determine the phloem unloading and invertase activity. We made about three-to four longitudinal sections and ten to twenty transversal sections per each root to investigate invertase activity localization. The figures show typical results. The boundary between the root zones was determined by morphometric analysis.

2.4. Biochemical Determination of Invertase Activity *in vitro*

The distribution of invertase activity was studied in root segments corresponding to different zones of the root: meristematic zone – apical 1.7 mm, elongation zone – 1.7-7 mm, the differentiation zone –10.2-15.5 mm from the root tip. The invertase activity was determined by the modified method described in [16].

Plant tissues were powdered in liquid nitrogen using the cooled mortar and pestle and was homogenized in buffer containing 50 мM Hepes, pH 7,5, 1 мM EDTA, 1 мM EGTA, 3 мM DTT, 5 мM MgCl$_2$, 0,5 мM PMSF. The homogenate was centrifuged at 15,000 g for 20 min. The precipitate was washed three times in homogenization buffer. The pellet and supernatant were dialyzed at 4 ° C overnight. Enzyme preparation was incubated in buffer at 30º C. To determine the activity of acid invertase, 0.1 M acetate buffer pH 4.7 was used. Alkaline invertase activity was determined in a K-phosphate buffer at pH 7.5. Sucrose concentration in the medium was 20 mM. The reaction was stopped by boiling in a water bath for 3 minutes. Immediately before boiling for stopping the reaction in probes for acid invertase activity, the solution was neutralized by the addition of 45 мM Tris. The amount of glucose formed during the hydrolysis of sucrose was determined by the glucose oxidase method (Fermognost, Blutzucker Test, VEB – Laborchemie Apolda). The reaction was stopped with 16 N H$_2$SO$_4$. Absorbance was measured at 530 nm (Spekol 11, Carl Zeiss, Germany).
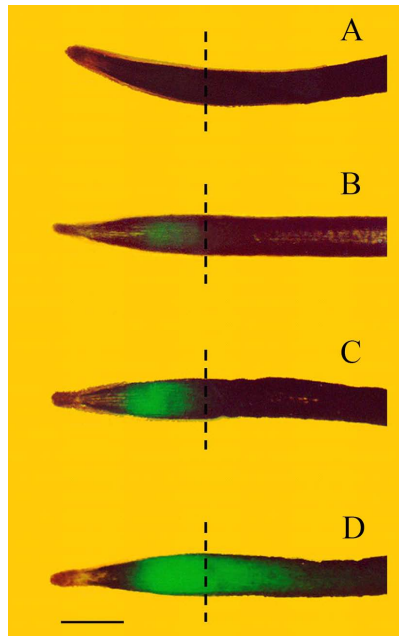
3. RESULTS

3.1. Localization of Phloem Unloading and Invertase Activity in the Root Tip

To elucidate the putative invertase role in phloem unloading, we estimated the enzyme activity and localization of phloem unloading in parallel. Since assimilates exit from the phloem into the root sink cells mainly via symplast [15, 17, 18], we used a fluorescent dye carboxyfluorescein (CF), a marker of symplastic transport to find out the localization of phloem unloading. A weak acid carboxyfluorescein diacetate (CFDA) was placed on seedling cotyledons; its undissociated form could penetrate the cells, where it was split by intracellular esterases, releasing CF. CF could not cross membranes and was transported in the phloem to the root tip, where it left the transport route, and was transported to the surrounding cells via plasmodesmata. The site of dye exit from the phloem was determined by its accumulation in

the root cells [4, 15].

Fig. (**1**) demonstrates localization of CF in the roots of melon seedlings. In 30 min after CFDA application to seedling cotyledons, we could observe the appearance of a fluorescing spot in the root tips; this indicated the exit of CF from the phloem. The fluorescing spot was approximately in the middle of the meristem. In this connection, we concluded that CF was unloaded from the phloem in the middle of the meristem. Intensity of fluorescence increased if the exposure time prolonged and spreaded into the basal half of the meristem and in the beginning of the elongation zone (Figs. **1B-D**).

Fig. (1). Localization of phloem unloading in the melon root tips. The emergence of the CF in the root tips in 0 (A), 1.5 (B), 4 (C) and 10 (D) hours after its application to the cotyledons. The dashed line indicates the boundary between the meristem and the elongation zone. The boundary between the root zones was determined by morphometric analysis. Bar – 500 μm.

Fig. (2). Staining of invertase activity in the root apex of maize (A), wheat (B) and melon (C). (A) – the longitudinal section of the root tip, in which the root cap was detached, (B, C) – the whole root tips. The dashed line on each root indicates the boundary between the meristem and the elongation zone. The boundary between the root zones was determined by morphometric analysis. Bar – 500 μm.

Invertase activity varied in different root zones. In the roots of maize (Fig. **2A**), wheat (Fig. **2B**) and melon (Fig. **1**), we observed the similar pattern of invertase activity. The activity was detected in the root cap (Figs. **2B**, **2C**, **3**). In the apical half of the meristem, invertase activity was detected only in the rhizodermal cells. In the apical part of the root tip, mainly occupied by meristem, the color was weak or there was no color at all. Approximately in the middle of the meristem zone, invertase activity appeared in the cortex and stellar cells. At a larger distance from the root tip, staining intensity indicating invertase activity increased and attained the highest value in the elongation zone. With further increase in the distance from the root tip, staining intensity reduced.
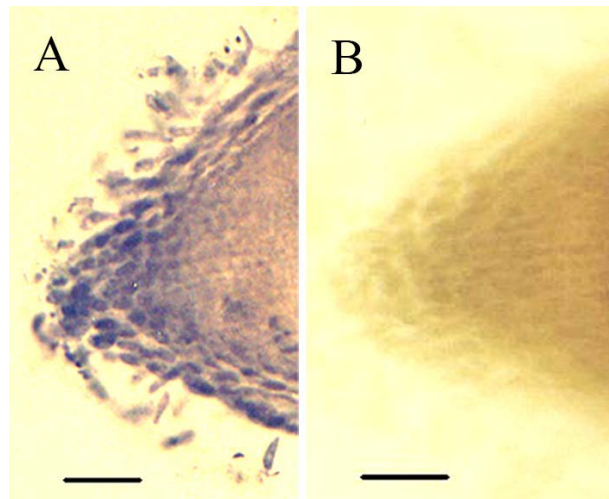

Fig. (3). Staining of invertase activity in the longitudinal sections of maize root caps. The reaction with (A) or without (B) sucrose in the incubation medium. Bar – 100 µm.
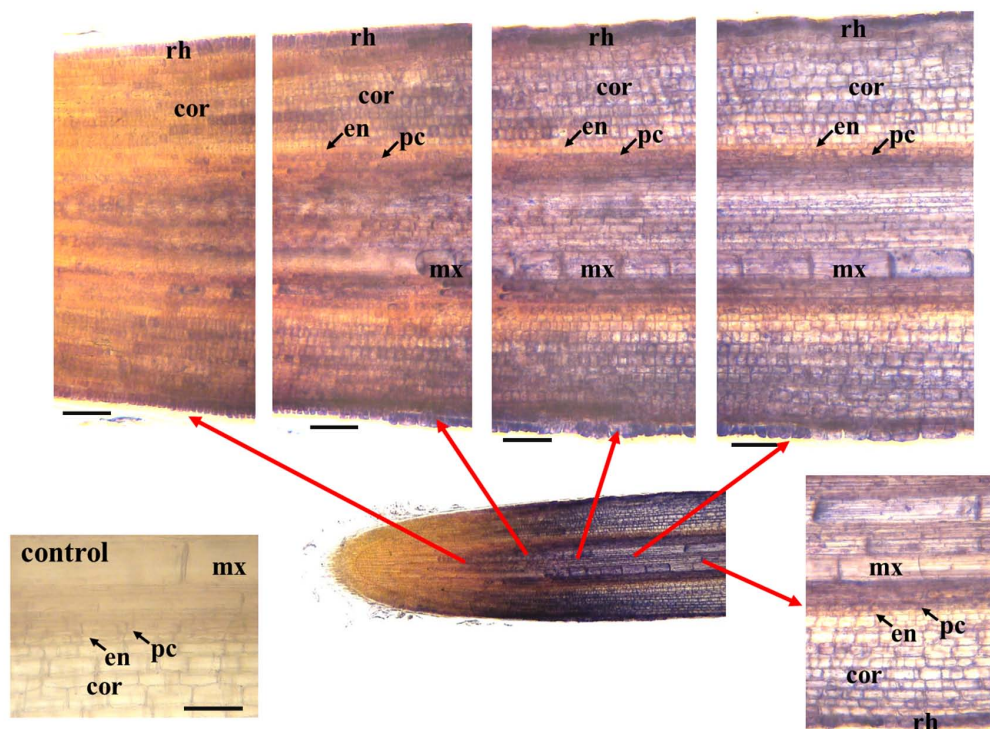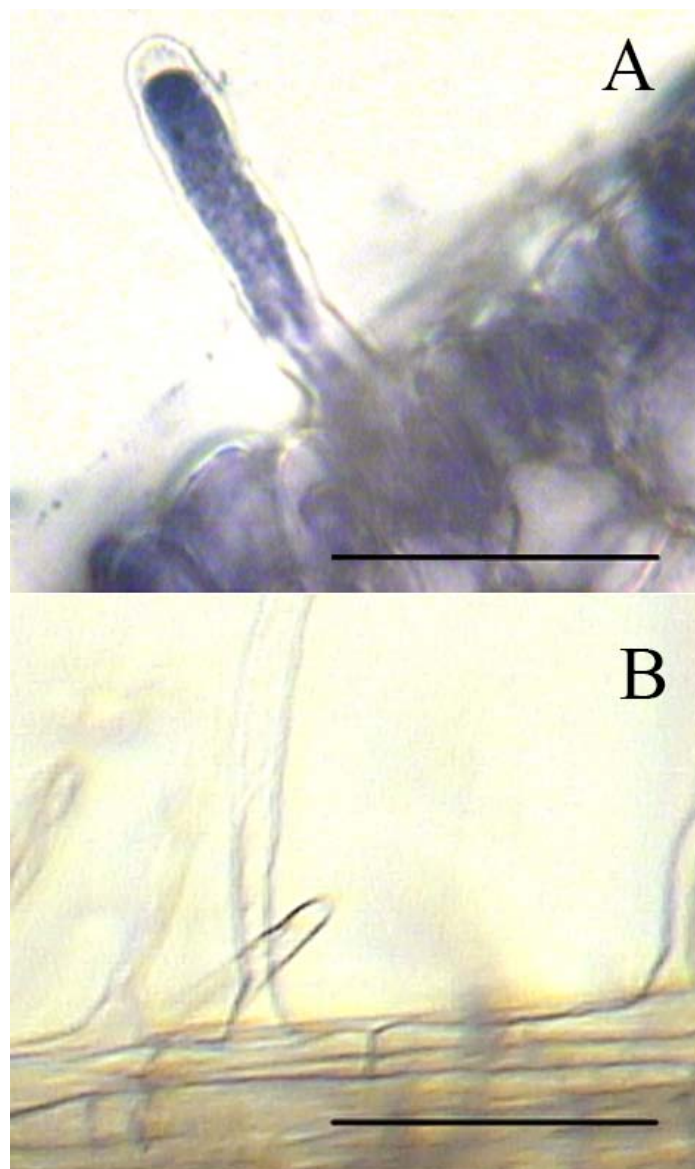

Fig. (4). Staining of invertase activity in the parts of the longitudinal sections of the maize root apex. The zone of transition from meristem to elongation is shown. At the bottom – the sections from the basal part of the elongation zone: in the left - the reaction without sucrose in the incubation medium (control), in the right – the reaction with sucrose. Bar – 100 µm. rh – rhizodermis, cor – cortex, en – endodermis, pc – pericycle, mx – metaxylem vessel.

3.2. Invertase Activity in Different Tissues of the Root Tip

The activity of invertase varied not only in different root zones but also in different tissues of the same zone. The distribution of invertase activity among different tissues was studied in more detail on the longitudinal (Figs. **3**, **4**) and transvers (Figs. **5**, **6**) sections of the maize root tip.

In the root cap, invertase activity weakly manifested in the cells adjoining to the root body, *i.e.*, in young dividing cells (Fig. **3**). This is in accordance with the absence of invertase activity in most of the cells of the root apical meristem (Fig. **2A**). Invertase was the most active in mature and even in detached root cap cells (Figs. **3**, **7**).

Invertase was first detected in the rhizodermis closer to the root tip than in other tissues (Figs. **3B**, **3C**, **4**), but it was absent near the boundary with the root cap. Staining of rhizodermal cells was brighter than of the other tissues cells (Fig. **6A**), and the root hairs were also stained (Fig. **5**).
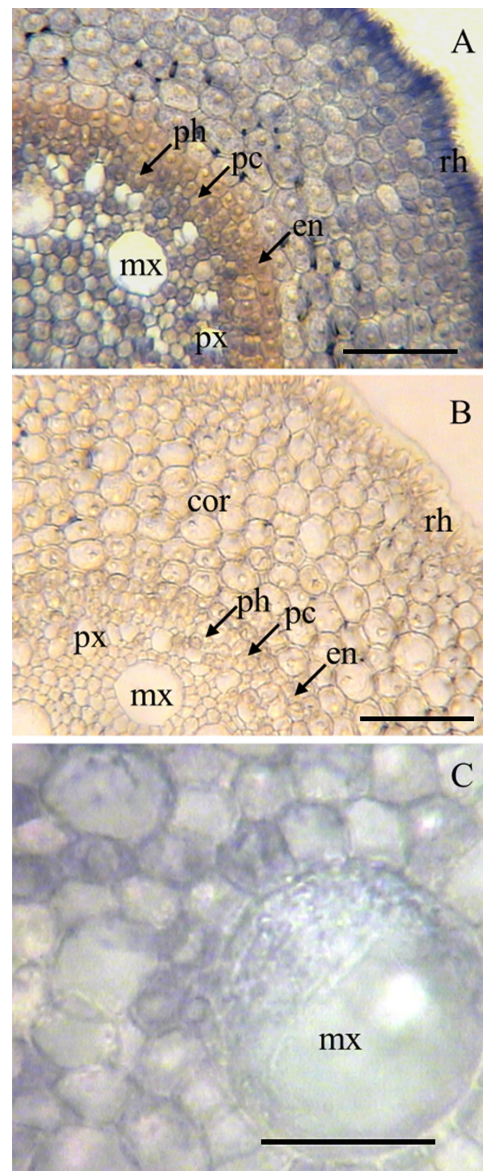

Fig. (5). Staining of invertase activity in a maize root hair. The reaction with (A) or without (B) sucrose in the incubation medium. Bar – 50 μm (A), 100 μm (B).
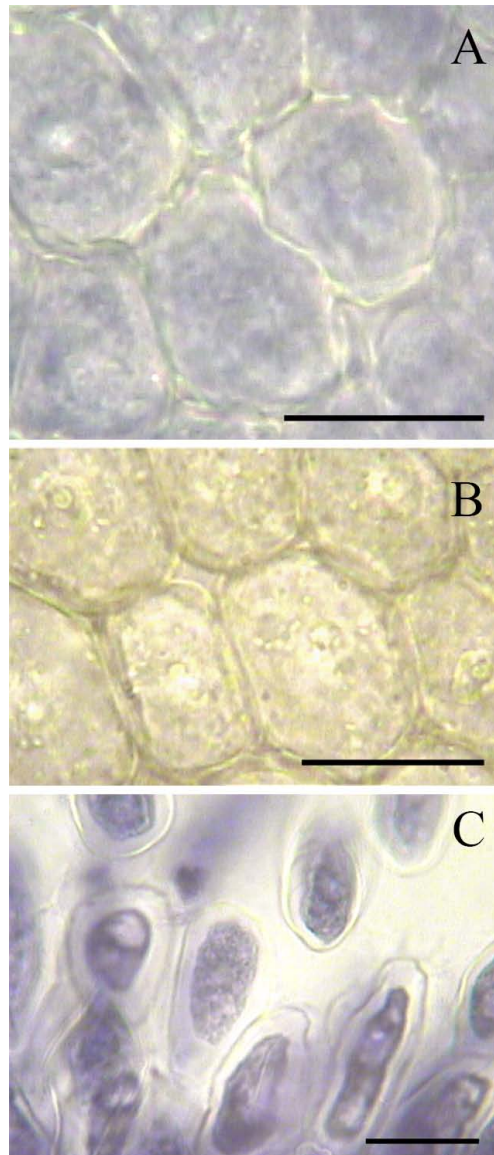
In the cortex and stele, the activity of invertase corresponded to the above described pattern of longitudinal enzyme distribution within the root. Invertase activity appeared in the basal part of the meristem and increased further away from the root tip and attained the highest values in the region of cell transition to elongation and in the beginning of the elongation zone (Fig. **4**).

In the cortex, we observed a radial gradient of staining intensity with the heaviest staining in peripheral cells adjoining to the rhizodermis (Fig. **6A**). However, even these peripheral cells were stained weaker than rhizodermal cells. The cell layer near the endodermis had the palest staining. It might be that such a pattern of invertase activity facilitated the creation of the sucrose concentration gradient facilitating its transport from vascular conducting bundles to the root periphery. The cells of endodermis were not stained at all and in the pericycle invertase, the activity was weak (Fig. **6A**).

In the cells of the stele, invertase activity was found in immature elements of the metaxylem (Fig. **6C**). The activity disappeared with the vessels differentiation. Similar situation was observed also for the sieve elements. In the beginning of the elongation zone, we observed both stained and unstained sieve elements of the protophloem. In the differentiation zone, no staining was observed in the sieve elements. Thus, in the conducting system, enzyme activity depends on the degree of its differentiation: mature cells of the metaxylem and sieve elements lost their enzyme activity, which was detected in immature cells.

Fig. (6). Staining of invertase activity on the parts of the transverse sections of a maize root tip from the region of the beginning of the elongation zone (A, B) and from the basal part of the meristem (C). The parts of the sections contain rhizodermis (rh), cortex (cor), endodermis (en), pericycle (pc) and stele (A, B) and a region of vascular cylinder (C). The reaction with (A, C) or without (B) sucrose in the incubation medium. Bar – 100 µm (A, B), 25 µm (C). ph – phloem, mx – metaxylem vessel, px – protoxylem vessel.

Fig. (7). Staining of invertase activity. (A, B) – cortex cells in the transverse sections in the basal part of the maize root meristem, (C) the border cells liberated from the maize root caps into the medium. All sections were treated with 0.7 M mannitol previously. The reaction with (A, C) or without (B) sucrose in the incubation medium. Bar – 25 μm.

3.3. Intracellular Localization of Invertase

As the blue color illustrated, the invertase activity is concentrated mostly inside the cells (Fig. **7**), we proposed that the activity was mostly the one of vacuolar invertase.

In the assays of invertase activity, nuclei, nucleoli, and slime around the root were usually not stained (Figs. **6** and **7**). The cell walls were practically colorless too. It was especially well seen in those sections, in which weak plasmolysis had been induced by treating with mannitol before incubating them to detect invertase activity (Fig. **7**). These results indicate that stained products of the reactions did not diffuse because nucleoli, cell walls, and the slime are known to be characterized by a high absorbing capacity. The above mentioned data and also the absence of staining in the control sections performed without sucrose in the incubation medium characterize the applicability of the method used for the detection of the enzyme activity. Some other authors use this technique to analyze the activity of invertase even in the root tips [14, 19].

3.4. Biochemical Determination of Invertase Activity *in Vitro*

The result of biochemical determination of invertase activity shows, that the vacuolar invertase was the most active

Table (**1**). The other two forms of invertase – cytoplasmic and, especially, apoplasmic – made a smaller contribution to the overall activity of the enzyme. This data is in coincidence with the result of histochemical analysis (Figs. **6** and **7**).

Table 1. Activity of the invertase forms in different maize root zones, mg glucose/g wet weight.

The Forms of Invertase	Invertase Activity in the Root Zones, mg Glucose/g Wet Weight		
	Meristem	**Elongation**	**Differentiation**
vacuolar	8,23±1,00	27,46±0,91	10,09±0,65
cytoplasmic	6,02±0,60	6,5±0,66	2,32±0,50
cell wall	0,62±0,13	1,9±0,30	0,53±0,15

Note: Values are mean from 3 experiments ± the standard error of the mean. In each experiment, about 20 roots were analyzed. To determine the statistical reliability, the Student's test was used ($p < 0.5$). The activity of the vacuolar invertase most sharply varied in different root zones.

The activity of this form of invertase in the zone of elongation was more than three times higher than that in the meristematic zone and more than 2,5-fold higher than in the differentiation zone. The differences between invertase activities in the meristem and in the differentiating zone are unreliable. The activity of cytoplasmic and cell wall invertases differed much weaker in the zones of different stages of development. The activity of the cell wall invertase was the least in comparison with the other forms of invertase. Similar data have been obtained previously for the roots of other plants [10 - 12].

4. DISCUSSION

Among three known invertase forms, cell-wall invertase is usually related to phloem unloading. Such notion arose on the basis of Eschrich hypothesis [3], according to which sucrose releasing from the phloem entered into the cell wall, where it is degraded by cell-wall invertase, and as a result, the sucrose concentration gradient is created. Hexoses produced in such a way could not be loaded back into the sieve element, the plasma membrane of which does not contain hexose transporters, and is absorbed by neighboring parenchymal cells. Such a scheme supposes the apoplastic pathway of phloem unloading, in which the cell wall invertase plays the main role. In fact, high activity of cell-wall invertase was demonstrated for the organs characterized by apoplastic unloading: seeds, stems, tubers and storage roots [1, 20].

Phloem unloading in the root tip is considered to be symplastic [15, 17]. Therefore, intracellular enzymes cleaving sucrose but not cell-wall invertase should facilitate this process. It is difficult to reach plasmolysis in the cells of the root tip. You can only see a hint of the beginning of plasmolysis after treatment with 0.7 M mannitol (Fig. **7A**). Meanwhile plasmolysis could be observed in the border cells around the root cap (Fig. **7C**). We found no significant blue staining and correspondently presence of invertase in the cell wall. But the intracellular localization of blue color predicts the occurrence of intracellular forms of the sucrose metabolizing enzyme, what confirms the symplastic transport to be the main pathway for sucrose in the root.

The method we used did not permit to evaluate unambiguously the ratio between vacuolar and cytoplasmic invertase activities in the cells within the region of phloem unloading. However, in view of the weak vacuolization of the cells in this region, and since the cell intracellular space is almost evenly stained in the invertase assay (Figs. **6** and **7**), we could suppose that both forms of intracellular invertase were active. This assumption is confirmed by data identifying the cytoplasmic invertase activity in the root cells of *Vicia faba* [19]. Indeed, biochemical analysis of various forms of invertase showed a small unreliable difference between the activity of the vacuolar and cytoplasmic invertases in the meristematic zone. The greatest difference was observed in the elongation zone, the place of intensive expansion of vacuoles (Table **1**).

Invertase, the sucrose metabolizing enzyme, should facilitate phloem unloading because its activity reduces sucrose concentration in sink cells. This makes steeper a gradient of sucrose concentration between terminal phloem elements and sucrose-consuming cells, which stimulates sucrose exit from the phloem [9, 20]. A comparison of the localization of symplastic phloem unloading and the longitudinal distribution of invertase activity in the root tip can be seen in (Figs. **1-2**). The apical boundary of the phloem unloading and the beginning of invertase activity are localized approximately in the middle of the meristem. This could indicate the interrelation between the phloem unloading and invertase activity. At the same time, apical boundary of the phloem unloading positioned closer to the root apex than the apical boundary of invertase activity appearance. That is to say, phloem unloading precedes the appearance of invertase

activity in the root tip. It could be assumed that sucrose received by the sink tissues induces invertase activity.

Maximum activity of invertase was found in the elongation zone (Fig. **4**, Table **1**), which is characterized by the increased formation of vacuoles. This is related to the fact that invertase converts one sucrose molecule into the two osmotically active hexose molecules. Their accumulation in the vacuoles increases osmotic pressure required for water income to the cells growing by elongation [21]. Therefore, just vacuolar invertase plays the most important role in processes related to cell elongation [22]. Besides osmotic action, sucrose and hexoses formed by the action of invertase are the components of cell signal systems [7, 23 - 25].

Thus, our data suggest that the flow of sucrose from the phloem can induce the activity of invertase. Changes in the content and ratio of sucrose and hexoses will increase osmotic pressure. The latter, as well as the involvement of sugars in the signaling system promotes cell elongation and root growth.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest. The authors did not receive any financial support.

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