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Original Article

Elevated Inulin Content in Chicory under Phosphorous and Iron Deficiency

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ABSTRACT

Inulin has been shown to possess probiotic properties and industrial applications. Chicory (*Cichorium intybus* L.) is the main commercial source of inulin throughout the whole world. However, the composition of the culture medium to grow chicory has a significant effect on both quality and the quantity of this active ingredient in the plant. Here, several sets of experiments were conducted in a completely randomized design under hydroponic culture. in vitro condition, to investigate the effects of phosphorus and iron fertilizer starvation on plant growth traits and also, the amount of active ingredient in chicory. Phosphorus was used at three different levels: 0.5, 1.25 and 2.5 mM while, the effect of iron treatment was investigated by removing iron from the MS culture medium and then compared to the control. The results showed that with increasing phosphorous consumption, elevated fresh and dry root weight was observed, however, iron starvation reduced root growth. In this study, the amount of inulin was significantly affected by these treatments while, the stress of phosphorus deficiency and also the elimination of iron increased the amount of inulin.

INTRODUCTION

Chicory (*Cichorium intybus* L.) is one of the most important herbs of the *Asteraceae* family which is known throughout the world as a traditional medicinal plant [1]. According to the researche's studies, this plant has antimalarial, hepatoprotective, antidiabetic, anti-inflammatory, tumor-inhibitory activities, and could improve skin barrier function [2,3]. Chicory contains a variety of ingredients including; polyphenols, fructans, inulin, cichoric acid, saccharides, organic acid, polyphenol, alkaloid, caffeic acid derivatives (chlorogenic acid, isochlorogenic acid, dicaffeoyl tartaric acid), triterpenes, sesquiterpene lactones (especially lactucin, 8- lactucin, glycosides), coumarins, flavones, esculin, etc, which in turn has a variety of applications [4,5]. Chicory roots is the most important source of industrial production of inulin due to the production of long and stable chains of it in the form of fructans [6].

Various biotic and abiotic stresses, such as widespread climate change, nutrition, salinity, pathogens, etc., affect the quantity and quality of chicory's active compounds such as inulin and some other secondary metabolites. Subsequently, the productivity as well as the medicinal value of the plant changes dramatically [7-9]. Among the stresses affecting secondary metabolites, the type and the amounts of minerals are noteworthy to mention. Until now, numerous studies have been reported to confirm the effect of minerals including phosphorus and iron on the quality and quantity of medicinal plants active ingredients. Phosphorus as a macronutrient, is a major component of biomolecules such as phospholipids and nucleic acids, which in the case of deficiency or defects in absorption, the plant encounters a decline in the yield [10]. In order to adapt and increase phosphorus reabsorption under phosphate stress conditions, plants develop sophisticated strategies including reducing shoot and root growth, developing root hairs, showing different physiological responses such as changes in glycolysis rates, primary and secondary metabolites production such as organic acids, sugars, and flavonoids [11, 12]. Studies on the effect of phosphorous deficiency in tea plant have shown a decrease in the synthesis of flavonoids,

phosphorylated metabolites and glutamates, although this deficiency raised glutamine levels and also root to shoot volume ratios [13]. Research conducted by Liu and colleagues (2018) has also shown that phosphorus deficiency can increase phenolic acid production and elevated antioxidant activity [14] and caused the accumulation of polysaccharides [15].

Iron is one of the micro elements that plays a key role in photosynthesis and crop production in plants, which its deficiency can be a limiting factor in plant growth [16]. Therefore, the amount of available and absorbable iron as well as iron homeostasis are important to increase the optimum yield of the plant [17-22]. Iron nanoparticles could increase the antioxidant enzymes activity, phenol and flavonoids levels as well as hairy root growth in Zarrin-Giah [23] . It was also shown that iron deficiency caused the accumulation of organic carbon in soybean root twice as much as control [24].

Chicory has been a focus of traditional and modern medicine for a long time, and the inulin derived from the chicory root is one of the most significant sources of inulin production in industrial and pharmaceutical units [25]. In this regard, nutrition is an important and effective factor on both the yield and inulin content of chicory [26, 27]. Phosphorus and iron are among the elements that influence metabolic activity as well as plant biological activity [28]. However, the effect of high or low concentrations of phosphorus and iron starvation on the production of inulin and its biomass has not been studied. Therefore, this study investigates the effects of these two minerals in achieving optimal nutritional requirements for inulin production with appropriate quantity and quality.

MATERIALS AND METHODS

Seeds of chicory (Pakan Bazr, Isfahan, Iran) used in this study were initially disinfected using 70% ethanol for 2 minutes and subsequently with 7% sodium hypochlorite for 5 minutes. Seeds were then washed three times with sterile distilled water, each time lasted 5 minutes. Seeds were then planted in solid MS medium (3% sucrose and 5% Agar) on a steel mesh plate. Containers were then placed inside a growth chamber, set at 25 ºC, for 16-hour light and 8-hour dark photoperiod. After the emergence of hypocotyls and primary roots, established plants on mesh plate were transferred to one-liter glass bottles containing liquid MS medium. Under the aforementioned condition, the explants were placed in a shaker incubator programmed for 16 hours of light and 8 hours of dark with temperatures of 23ºC while shaking at 5 rpm. In order to provide the fresh nutrients, the culture medium was changed every three days. After one week, phosphorus and iron treatments were investigated to see their effects on plant growth, quantity and quality of inulin. For this, plants were transferred to MS medium with changes in the amounts of Fe and Pi. In all cases, MS medium (1.25 mM KH_2PO_4) was considered as control. Phosphorus was used at three levels of 0.5, 1.25 (normal) and 2.5 mM. The effect of iron treatment was investigated by removing iron from the MS culture medium compared to the control. Experiment was continued for 2 weeks using different levels of phosphorus and iron. In this test, 9 replicates out of total of 18 replicates, were used for phenotypic measurements and the rest of the replicates were examined for the subsequent analysis of the extract production. Next, data were analyzed using a completely randomized block design with 9 replications.

Phenotypic Measurements

Two weeks after the treatments, the roots were separated from other parts of the plants and washed thoroughly with distilled water. Root length, fresh weight and dry weight were measured as the root phenotypic traits. To measure dry weight, roots were incubated in oven for 12 h at 72 ºC.

Production of Plant Extract

The hairy roots were chopped and diluted with sterilized distilled water (1:5). Acidity was set at 5 using hydrochloric acid and then the mixture was incubated for 1 hour in a water bath at 60 ºC. After incubation, the liquid and solid phases were separated using a 0.45-micron filter. The suspension was centrifuged for 5 minutes at 2 rpm. Then 1 ml of the supernatant was taken and water was added to a volume of 100 ml which was used in subsequent steps [31].

Total Sugar Content

Phenol sulfuric acid method was applied to measure the total sugar content in the water-soluble components, extracted from chicory roots [29]. Glucose was used as the standard curve of total sugar content (Fig. 1A).

Reduced Sugar Content

For the measurement of the reduced sugar, Baldini *et al* (2004) method was used. For this purpose, Di nitro salicylic acid (DNSA) reagent was prepared by solving 25 g sodium potassium tartrate, 1.6 g sodium hydroxide and 1 g of nitro salicylic acid in 100 ml H_2O. Then 2.5 ml of reagent was added to 0.1 ml diluted extract (1%) and boiled in a water bath at 90 °C for 10 min. After cooling the samples, 2.4 ml of distilled water was added to each sample and finally the absorbance was read at 530 nm [30]. Glucose was used to prepare the standard curve of reduced sugar content (Fig. 1B).

Inulin Content

In order to measure the amount of inulin in chicory roots, the reduced sugar content was subtracted from the total sugar content and the inulin extraction efficiency was calculated using the following equation [30].

Inulin extraction efficiency (%) = ((amount of inulin × volume of extract)/root dry weight) × 100

The inulin degree of polymerization

The average degree of inulin polymerization is obtained by dividing the weight percentage of total sugar content by the weight percentage of reduced sugar, which is an important factor in determining the quality of extracted inulin [31].

Data Analysis

Data analysis of variance was done applying SAS software ver. 9.4. Correlation analysis was done using Performance Analytics package in Rstudio. GGE biplot was also drawn applying GGE biplots package in Rstudio software.

RESULT AND DISCUSSION

Evaluation of fresh weight of seedlings in different levels of P and Fe demonstrated significant differences between different treatments (Table 1). Using higher levels of phosphorus significantly increased seedling fresh weight under *in vitro* conditions. Regarding the dry weight of seedlings, a decreasing or increasing P and Fe trend similar to the fresh weight was perceived. On the other hand, root length, showed completely different pattern and lower amount of phosphate could increase root length meaningfully. In this case, adding higher amounts of phosphorus and removal of Fe from the medium ended up in decreased root length. However, total sugar content, reduced sugar and polymerization degree were not affected by the abovementioned treatments, while the inulin content was significantly affected.

Two-sided correlation analysis between traits by Pearson method showed that both fresh weight and dry weight had a meaningful negative correlation with inulin content (Fig. 2). Based on the results, total sugar content showed a meaningful positive correlation with the percentage of inulin and, average degree of inulin polymerization (DPn). There was also a significant positive correlation between the inulin content and the reduced sugar, and total sugar contents and also DPn (Fig. 2).

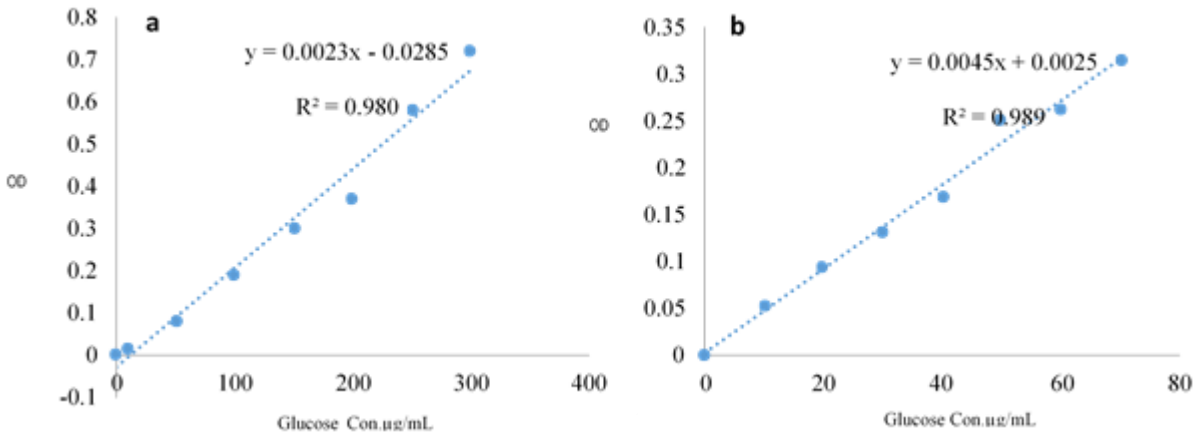

Fig. 1 Standard calibration curves for glucose. A: Total sugar content. B: Reduced sugar content.

Table 1 Analysis of variance on the effect of different levels of P and Fe on chicory root, and the mean of measured traits

Traits	Treatments Pr(>F)	Mean			
		0.5_mM_phosphate	1.25_mM_phosphate_control	3.75_mM_phosphate	-Fe
Root fresh weight	5.72e-05 ***	245.44±21.15 b	337.67±31.80 a	403.78±31.52 a	222.11±12.46 b
Root dry weight	7.73e-05 ***	19.22±2.19 b	30.00±3.09 a	35.11±3.51a	16.56±1.45 b
Root length	0.00248 **	8.22±0.4403 a	7.90±2.6333 ab	7.19±0.1911 bc	6.31±0.2423 c
Total sugar	0.776 ns	0.367±0.050 a	0.332±0.043 a	0.322±0.0050 a	0.433±0.145 a
Reduced sugar	0.0559 ns	0.1939±0.0039 b	0.1905±0.0051 b	0.1989±0.0047 ab	0.2096±0.0063 a
Inulin%	3.94e-05 ***	1.97±0.5459 b	0.48±0.1192 c	0.34±0.1317 c	3.38±0.5922 a
Polymerization	0.849 ns	1.72±0.5019 a	1.42±0.3879 a	1.26±0.4841 a	2.23±1.45 a

Mean of values ± standard errors of the respective traits. Values followed by different letters are significantly different according to the Duncan's Multiple Range Test at $p< 0.05$.

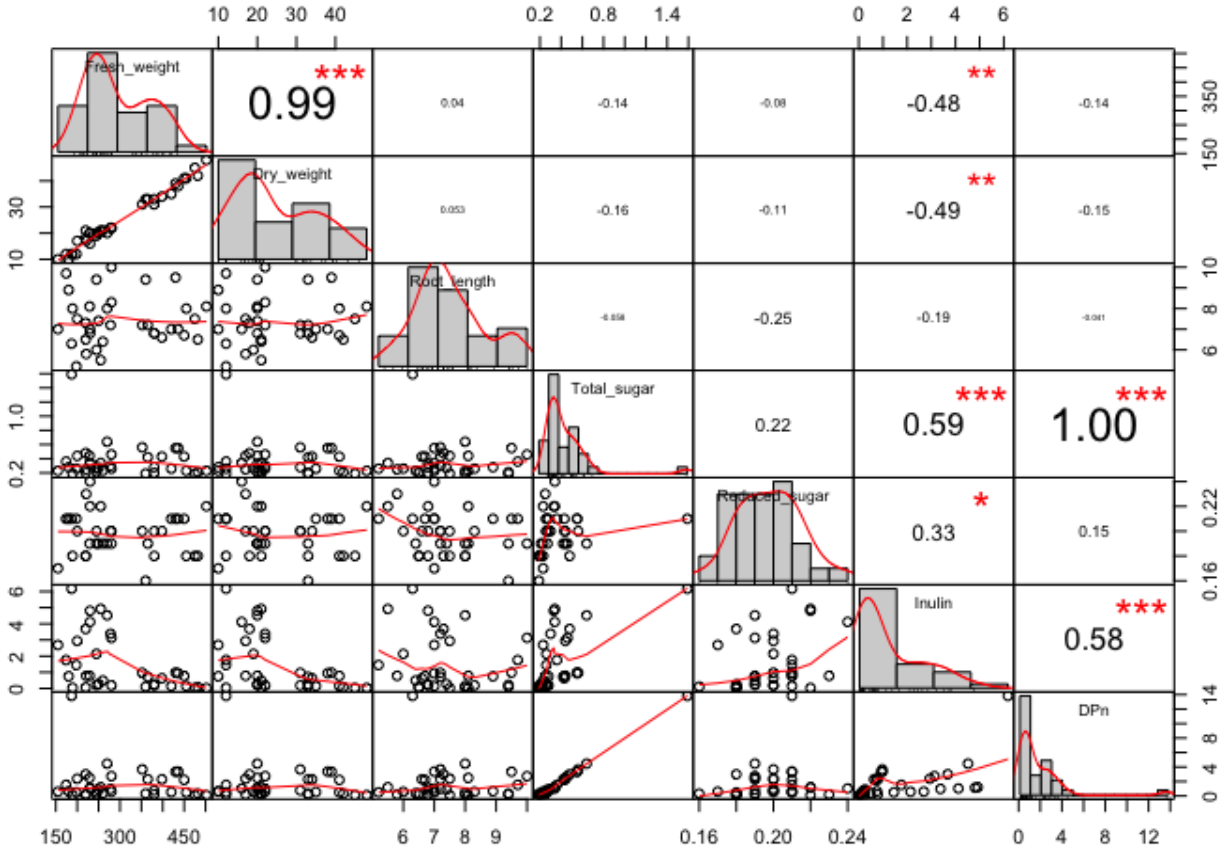

Fig. 2 Two-sided correlation analysis between traits by Pearson method

To provide a visualized means of the pattern for the dataset, a GGE biplot for all measured traits have been drawn that explains 99.50% of the total variation (Fig. 3). It is obvious that removal of iron from the medium culture was associated with the increased total sugar, inulin content and DPn, while increasing phosphorus content (up to 3.75 mM) increased dry weight and fresh weight of seedlings. Phosphate has been introduced as an important parameter affecting cell growth and metabolism and is involved in energy metabolism and biosynthesis[32]. Studies show that phosphorus can have a significant impact on the production of secondary metabolites as well as biomass growth under *in Vitro* conditions. For example, the improvement of growth parameters along with the decrease in the accumulation of certain secondary metabolites such as phenolic acids and tanshinones in the hairy roots of the two plants *S. miltiorrhiza* and *S. castanea* have been reported after increasing phosphate in the culture medium [14]. Another study presented that phosphorus was an effective factor in the production of ginsenosides in the hairy root culture of *Panax quinquefolium*. The results of this study showed that the biomass production increased with increasing phosphorus concentration in the culture medium, while production of secondary metabolites decreased[33].

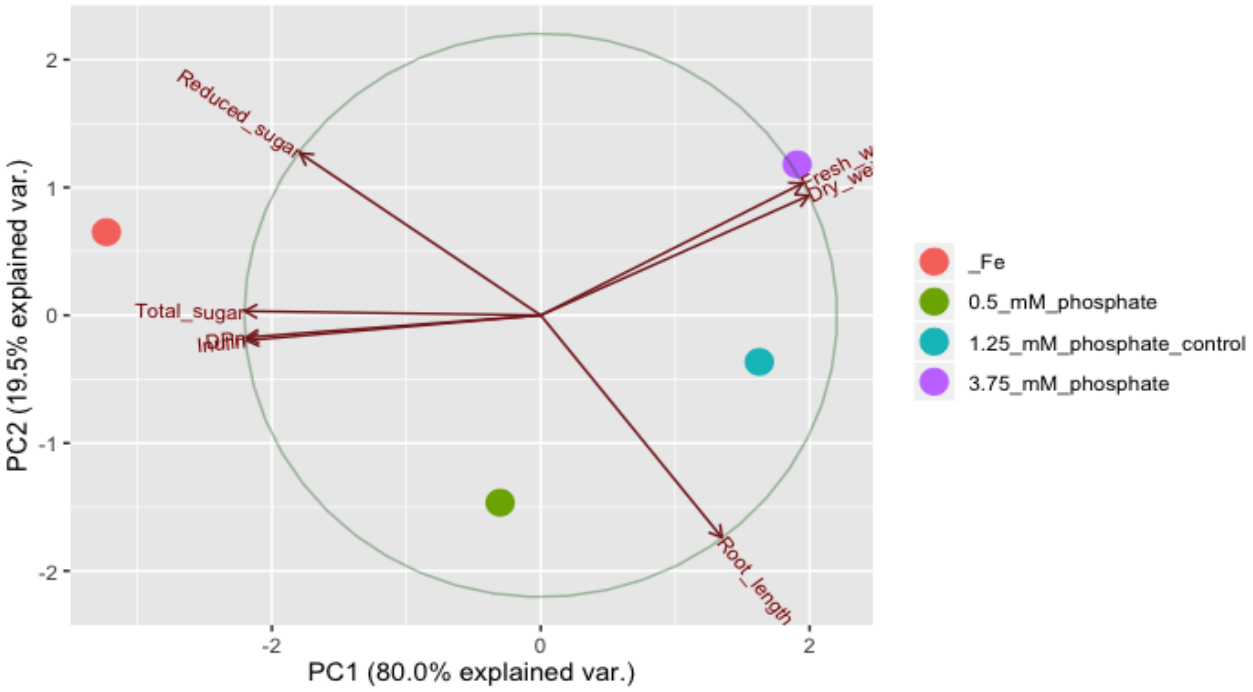

Fig. 3 GGE biplot for all measured traits, a visualized means of the pattern for the dataset. The first and second component explained about 99.5% of total data variance. Variables as points are shown in the space of principal components

Generally, the results demonstrated that removal of Fe from the medium culture could increase the inulin content (3.38 ± 0.5922) by 604% compared to the control (0.48 ± 0.1192), whereas phosphorus starvation increased the inulin content (1.97 ± 0.5459) by 310% in comparison to the control. On the other hand, a three-fold increase in phosphorus levels compared to the control, reduced inulin levels by 29%.

In a study on hairy root of chicory by the authors, it was shown that increasing phosphorus in the culture medium leads to higher fresh weight and lower amount of inulin [34]. The results of our study showed that inulin accumulation with polysaccharide structure under phosphorus deficient conditions, was associated with a decrease in biomass production, although under these circumstances an increase in root length was also observed. This phenomenon may be related to the multiple roles of phosphorus in the plant photochemical reactions. Studies have shown that many changes in phosphorus deficient conditions including increased cluster roots, decreased root content, hydraulic conductivity and ability to absorb and transfer to the stem, are related to the amount and function of some hormones like cytokinin [35]. Nutritional deficiencies disrupts the balance of hormone production, thereby affecting plant growth and stress tolerance[36]. Since the synthesis rate drops in phosphorus deficiency, the growth of aerial parts slows down, resulting in reduced photosynthesis and transfer of photosynthetic materials[37]. Reducing the cell expansion, preventing leaf growth, and shortening plant heights in conditions of phosphorus deficiency can also be attributed to the reduced hydraulic root conductivity[38]. On the other hand, the accumulation of photosynthesis products, including sugar in leaf and root tissues, is also expected in conditions of phosphorus deficiency which could be due to the reduced photosynthesis and consumption of photosynthetic products [16]. The source of root-soluble sugars is the leaves, which under condition of phosphorus deficiency, their transmission through the phloem to the roots increases and causes the spread of root system in some species [39]. In our study, the increase in inulin in roots was accompanied by an increase in root length. These results are consistent with reports from other plant species, including cluster bean [37]. It should be noted that the reduction of transpiration in plants with phosphorus deficiency can be effective in maintaining the water balance of these plants, because under these conditions, the hydraulic conductivity of the roots and the ability to absorb and conduct water in this tissue reduces [40]. On the other hand, the tendency to accumulate carbohydrate under phosphorus deficiency conditions can be one of the plant's responses to non-biological stresses, because carbohydrates, along with osmolytes, play a role in protecting cells under stresses [39, 41]. In

many species, including sugar beet, beans, and wheat, increased root's sugar-soluble have been reported along with increased root growth in phosphorus deficiency treatments under *in vitro* condition [39]. Increasing root length in phosphorus deficiency conditions is one of the plant's strategies to increase the absorption capacity for facing deficiency of the macro element. Phosphorus is one of the elements that is mainly accessed through diffusion and as a result, root growth and development increases its access to higher volume of soil and more absorption of it [42]. Studies have shown that further transfer of photosynthetic products to the roots in the absence of phosphorus causes roots to grow higher in many plants [39]. The results also show that increasing the concentration of soluble sugars in the roots, under the phosphorous deficiency in white lupin and Arabidopsis, act as signal-transduction pathways and stimulate the spread of roots [43]. Other studies have found similar results in other plants such as sugar beets, wheat, and beans [44].

Iron is also one of the most common elements in the earth's crust and plays an important role in metabolic processes such as respiration, photosynthesis, and DNA synthesis [28]. Iron, on one hand, plays an important role in reducing the destructive activity of free radicals and ROS [45], and also plays a vital role in the transmission chain of cytochromes [28, 46]. Therefore, if the iron deficiency restricts the cytochrome pathway and accumulate ROS in the mitochondria, a reduction in biomass production will be expected [47]. When the plant goes through growth retardation, it does not use its carbohydrate reserves and accumulate them in the cells [48]. Our results showed that iron starvation caused a significant accumulation of inulin compared to the control, but under this condition the biomass showed a significant decrease, which could be related to the role of iron in the plant's respiratory system. Interestingly, the root also showed the same pattern [34]. Studies on soybean root under iron deficiency condition have shown a doubling of organic carbon in the roots of this plant compared to control [24]. Also, a study of sugar beet root showed that iron deficiency caused more than 50 times increase in the carbon content of cytosol, which occurs through increased phosphoenolpyruvate carboxylase activity [49]. This phenomenon indicates that during iron depletion, coordinated changes occur in the genes expression pattern involved in the regulation and use of sucrose in the roots [50].

Analysis of bilateral correlation between treatments by Pearson method showed that there is a positive and significant correlation between total sugar with inulin production percentage and the average degree of inulin polymerization (Fig. 2). This correlation can be attributed to the equilibrium relationship between available sugar, inulin synthesis and the average degree of polymerization in the plant, because inulin synthesis requires two different enzymes, initially Sucrose 1(F)-fructosyltransferase enzyme (1-SST, EC 2.4.1.99) forms a trisaccharide 1-kestose and a free glucose , then in a reversible reaction, the enzyme 1,2-beta-D-fructan 1(F)-fructosyltransferase (1-FFT, EC 2.4.1.100) lengthen the chain by transferring units of fructose (fructose radical) to 1-ketosis (or a larger inulin) from another inulin molecule. Reducing the activity of the 1-SST enzyme causes deficiency of 1-ketosis, thus, the transfer of fructosyl units from l-chain inulin and its conversion to 1-ketosis, will take place. Therefore, the total sugar content down regulates the activity of enzymes, but up-regulates the production of inulin and the average degree of polymerization in proportion to total sugar content [40].

A general conclusion is that chicory, like many other plants, has undergone a wide range of physiological and biochemical changes to be able to conquer phosphorous deficiency conditions, including increasing total sugar, lowering reduced sugar content, and increasing the degree of inulin polymerization. However, the plants have adopted strategies to increase access to phosphorus and tolerate stresses caused by its deficiency, which were observed in the form of reduced biomass, elevated inulin content and increased root length. Iron starvation, on the other hand, has affected many of the vital activities of chicory and has reduced root length. However, according to previous studies, the removal of iron induces a set of gene expressions to regulate the use of sucrose in the roots, and may be a reason for the increase in the accumulation of sugar, inulin and the degree of polymerization in the roots of chicory in iron removal conditions.

Conflict of Interest

The authors declare that they have no conflict of interest.

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