Archives of Biological Sciences

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Ćosić T, Savić J, Raspor M, Cingel A, Ghalawnji N, Vinterhalter B, Ninković S. Effects of different types of sugars and plant growth regulators on kohlrabi seedling growth and development *in vitro*. Arch Biol Sci. 2020;https://doi.org/10.2298/ABS200622029C.

Effects of different types of sugars and plant growth regulators on kohlrabi seedling growth and development *in vitro*

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Received: June 22, 2020; **Accepted:** July 1, 2020; **Published online:** July 14, 2020

Abstract: Kohlrabi (*Brassica oleracea* var. *gongylodes*), with its edible stem tuber formed at the base of the plant stem, presents a valuable source of nutrients. The potential effects of plant growth regulators (PGRs), as well as various concentrations of different sugars on the *in vitro* development of kohlrabi were studied. Ten-day-old kohlrabi seedlings were cultivated *in vitro* for 5 weeks at 18±2°C on half-strength MS media containing different concentrations of carbon source such as sucrose, fructose, glucose, xylose and mannitol, combined with or without specific plant growth regulators (N^6-benzyladenine (BA), gibberellic acid (GA_3), 2,3,5-triiodobenzoic acid (TIBA)). Results showed no tuber formation in all treatments, but growth and development of treated kohlrabi seedlings was significantly affected in a distinctive manner, with a variety of morphological traits being altered in comparison to matching controls.

Keywords: kohlrabi tissue culture; sugars; plant growth regulators; morphogenesis, *de novo* shoot formation

INTRODUCTION

A wide range of species from the Brassicaceae family is recognized for their contribution to human and animal nutrition [1]. Among them, *Brassica* vegetables are the most prevalent. They contain little fat and are good source of vitamins, minerals and fiber. Kohlrabi (*Brassica oleracea* var. *gongylodes*) is generally considered a less traditional vegetable in the garden, however it is widely grown for the edible tuber formed at the lower part of the plant stem.

The capability of kohlrabi to accumulate assimilates in the aboveground stem is a distinctive feature of this plant species, making it an interesting subject in studying the mechanism of stem tuber formation. This process in kohlrabi normally takes place between the 3rd and 5th nodes. It has been observed that the process begins with rapid divisions of the central pith parenchyma cells [2]. These cells are capable of preserving meristematic activity for a long period. Subsequently, zones of dividing cells expand higher and laterally, forming new centers of active division. However, the mechanism of tissue swelling in kohlrabi, as well as regulation of this process, are still poorly understood.

According to literature data, the development of plant storage organs is profoundly based on the supply of assimilates from carbohydrate sources [3]. The process of photosynthesis and

sink organ requirements are highly coordinated through metabolic regulation in which specific sugar signalization plays a special role in different parts of plants.

The dominant form of sugar produced during photosynthesis is sucrose, which is also the main transport sugar form in plants [4]. This disaccharide is translocated via the phloem to storage organs such as young leaves and heterotrophic organs. Sucrose from these organs is used either for growth or it accumulates in the form of sucrose or starch, thereby acting as a regulating factor in the development and metabolism of storage organs [5].

In plant tissue culture media, sucrose is an extensively used carbohydrate resource and previous studies have shown that it presents the most favorable carbon source [6]. Furthermore, there are also reports of other sugars used as suitable carbon sources for *in vitro* culture of different plants [7,8]. Alcohol sugars, such as mannitol, were shown to be good substitutes for sucrose *in vitro*, serving as a rich carbon source and as an osmotic regulator as well [9].

Aside from being an appropriate carbon source for utilization by plantlets, an overload of sucrose can be converted to starch in developing storage organs. Several studies have provided evidence that increasing sugar concentration in the growth medium can promote the formation of storage organs such as microtubers in *Solanum tuberosum* [10] and *Xanthosoma sagittifolium* [11], the microrhizome in *Zingiber officinale* [12] and bulbs in *Allium cepa* L. [13]. Sugars have also been shown to function as signaling molecules whose transduction pathways affect metabolic and developmental processes [14]. Studies suggest that certain effects of sugar on the growth and development of plants are products of interaction in hormonal regulation [6,15].

Investigations of the effect of plant hormones on kohlrabi stem swelling are rather scarce [2]. Previous results have shown the possible involvement of plant hormones like gibberellins, cytokinins or auxins in controlling the development of stem tubers in different species [16-19]. The development of a swollen stem in stem mustard (*Brassica juncea* var. *tsatsai*) is a complex process, involving plant hormones such as GAs and cytokinins [18]. The interaction between sucrose and GAs in tuber formation was investigated and GA_3 was reported to increase the flow of assimilates into developing organs [16,20]. Studies have indicated that the translocation of assimilates from source to storage organs might be under the control of plant hormones [21]. Furthermore, it has been shown that plant hormones also regulate the activities of invertases, enzymes that catalyze the hydrolysis of sucrose into hexoses [22,23], and the development of storage organs and assimilate accumulation is stimulated by changes in the hexoses/sucrose ratio [24].

Over the last years, extensive research was conducted in order to identify the genes that underlie the induction and development of storage organs in various food crops such as potato [25], carrot [26], turnip [27] and radish [28]. Recently, a comparative transcriptomics study was undertaken in which the patterns of gene expression of tubers in species of *Brassica oleracea*, including kohlrabi, and *Brassica rapa*, were compared; the obtained findings revealed that genes and pathways that participate in *Brassica* tuber development include lipoxygenases, sucrose biosynthesis genes, as well as auxin metabolic genes [29].

The impact of PGRs on *de novo* organogenesis in kohlrabi has been recently studied, with particular attention given to the cytokinins [30,31]. The most frequently used cytokinin for inducing plant regeneration *in vitro*, including the *Brassica* genus [32-35], is N^6-bezyladenine (BA). This is consistent with our previous findings showing that the frequency of shoot regeneration was highest on nutrient medium with BA or TDZ, when intact seedlings were used as a starting material for induction of *de novo* organogenesis [30].

For decades, *in vitro* culture has proven to be a convenient method for investigating physiological processes in plants. The use of this type of biotechnological procedure offers prospects for establishing a stable and reproducible model system for studying the mechanisms underlying the storage of assimilates in plant stem, such as in kohlrabi stem swelling, including hormonal interactions that initiate this process [2].

This study was conducted to investigate the effects of different concentrations of various sugars as well as PGRs on *in vitro* growth and development of kohlrabi, with emphasis on the possible induction of stem swelling as a step in the elucidation of this process. Investigation of these interactions in kohlrabi has not been conducted to date.

MATERIALS AND METHODS

Plant material and culture conditions

Seeds of *Brassica oleracea* var. *gongylodes*, cultivar Vienna Purple, were surface sterilized with a preliminary 5-min submersion in ethanol 70%, followed by 30 min in 30% commercial bleach (4-6% NaOCl) with a drop of detergent (Fairy; Procter and Gamble, London, England) and a final thorough rinsing of the seeds using sterile distilled water. Seeds were aseptically transferred to 90 x 15 mm Petri dishes with a hormone-free basal medium containing Murashige and Skoog (MS) mineral salts [36], Linsmaier and Skoog (LS) vitamins [37], 3% sucrose, 100 mg/l myo-inositol and 0.6% agar, for germination.

Single 10-day-old seedlings were then placed in tubes (Ø 18 mm) with diversely enriched half-strength MS (1/2 MS) media and cultivated *in vitro* for 5 weeks at 18±2°C and a 16 h light photoperiod. Different concentrations of several types of sugar such as sucrose, fructose, glucose, xylose and mannitol, were added to the media combined with PGRs (N^6-bezyladenine (BA); gibberellic acid (GA_3); 2,3,5-triiodobenzoic acid (TIBA; Supplementary Table S1). All media were adjusted to pH 5.8 prior to autoclaving at 114°C and 80 kPa for 25 min. In all experiments, 10 seedlings were used per treatment, and each treatment was repeated three times.

Data collection and statistical analysis

Data were collected after 5 weeks of different treatments. All percentage-data were subjected to angular transformation ($\arcsin\sqrt{X}$) prior to analysis. After analysis, data were subjected to inverse transformation for presentation. Statistical significance was determined by analysis of variance (ANOVA) using SAS software (SAS Institute, 2004. SAS/STAT, ver. 9.1.SAS Institute Inc., Cary, NC, USA). The mean values were separated by Fisher's LSD post-hoc test at the 5% level of probability.

RESULTS

The effect of applied carbohydrates alone or in combination with plant growth regulators on kohlrabi *in vitro* growth and development was investigated. Results demonstrated different responses regarding morphological characteristics, such as plant height, the appearance of the leaves, stems and roots, as well as callus formation and shoot regeneration (Fig. 1A-F).

Plantlet height

Plantlet height was significantly decreased with application of higher concentrations of sucrose (Fig. 2), particularly when 9% sucrose was added to the growth medium, compared to control kohlrabi grown on standard 3% sucrose medium which exhibited normal morphology (Fig. 1A).

The addition of PGRs to the growth medium enhanced the height depression effect in the case of B type media, as in B9, where the combination of high sugar concentration and 1 mg/L BA + 1 mg/L TIBA induced the most significant maximum difference (Fig. 2). Further addition

of GA_3 in C type media reduced the influence of BA and TIBA, so that the recorded plantlet height was more similar to the control cultivated without PGRs (Fig 2).

When kohlrabi seedlings were grown on 9% sucrose, the addition of 1% glucose did not affect plantlet height. However, S2 and S3 types of media that contained 1% of xylose and 1% of xylose and fructose, respectively, had a negative impact according to ANOVA (Fig. 3A).

Mannitol also displayed a negative effect on plantlet height compared to the control, with the strongest effect expressed at concentrations of 6% and 9% (Fig. 3B).

Leaf, stem and root morphology

Leaf desiccation was observed in kohlrabi plantlets grown in the presence of PGRs (Fig. 4A). Almost all types of PGR-containing media had a similar effect, with about 40% of plantlets displaying leaf dryness. The exception was C9 medium, reaching the highest percentage of almost 80% of affected plantlets.

The addition of mannitol to the growth media ultimately induced severe damage to the plantlet leaves, leading to complete desiccation in the M9 treatment (Fig. 1B). Furthermore, mannitol and a high concentration of sucrose in combination with glucose, xylose and fructose (M and S types of media, respectively) induced an alteration in leaf color from the normal green (Fig. 1A) to dark blue-green (Fig. 1C).

All treatments, except control plantlets grown on K3 medium, exhibited a distinct percentage of plantlets showing loss of stem coloration after five weeks of culturing (Fig. 1D). At higher concentrations of sucrose (6% and 9%) in combination with PGRs, a larger number of plantlets exhibited stem discoloration (up to 70%; Fig. 4B).

In addition, the highest percentage of plantlets with loss of stem coloration was noted in S1 medium (Fig. 5A). When kohlrabi plantlets were grown on M-type media, the values were similar to those recorded for B and C types of media, with no significant differences with regard to increasing concentrations of added mannitol (Fig. 5B).

All plantlets grown on S, B and C types of media exhibited changed root appearance, from normal white (Fig. 1A) to brownish-yellow (Fig. 1C), with a total absence of root formation in the majority of plantlets cultured on B6 and B9. M6 and M9 treatments significantly affected root growth in the plantlets as well (Fig. 1B).

Callus formation and *in vitro* regeneration

Development of the callus on the stem bottom of the kohlrabi plantlets was observed in all treatments, excluding K3. The highest number of plantlets forming a callus was recorded for media containing PGRs (Fig. 6A). Shoot regeneration via the process of indirect organogenesis was primarily documented in plantlets grown on the media enriched with PGRs (Fig. 1E, F), with the best response observed in C3 plantlets that were treated with 1 mg/L BA, 1 mg/L TIBA and 2 mg/L GA_3 in medium containing 3% sucrose (Fig. 6B).

DISCUSSION

Plants use carbohydrates as signaling molecules, which integrate the effect of environmental conditions and plant developmental programs controlled by hormones [38-40]. A higher sucrose concentration (6-12%) in growth media was shown to promote tuberization in potato [16,41,42] and *Gloriosa superb* L. [43], and the induction of bulbs in garlic [44] and shallot [45]. Recent findings [29] have confirmed that genes regulating sucrose biosynthesis are implicated in the process of tuber development in kohlrabi. However, all carbon sources that were applied in our experiments failed to induce tuber formation in kohlrabi plantlet stems after 5 weeks of *in vitro* culturing.

Cytokinins and gibberellins have also been associated with tuber formation in plants [46-48]. They were shown to impact photosynthate transport and the partitioning of assimilates [49]. However, in some *in vitro* model systems such as kohlrabi in our study, no significant effects of these two groups of PGRs on stem swelling and tuber formation were detected. Despite the absence of stem thickening, the results presented here show that sugars alone or in combination with growth regulators influenced kohlrabi growth and development *in vitro*, ultimately leading to diverse responses in terms of morphological features such as plant height, the appearance of leaves, stems, roots, as well as callus formation and shoot regeneration.

Plantlet height was decreased with higher concentrations of sucrose (in particular at 9%), compared to control kohlrabi. Different concentrations of sugars, such as sucrose, have previously been shown to affect normal growth and development of plant tissues and organs such as leaves, tubers and adventitious roots [50,51]. These results and many other observations highlight the importance of sugar signaling pathways in the regulation of plant growth [52,53]. Plant growth depends not only on available resources, but also on developmental and physiological signals that require the monitoring of resource levels. Plants that grow more slowly set aside their resources, possibly as reserves for unfavorable conditions [52].

It was suggested that the necessary conditions for kohlrabi stem swelling included the presence of cytokinins and a low level of auxin [2]. The application of BA aborted stem elongation and accelerated swelling. On the other hand, GA_3 applied with BA increased both stem height and thickness, with or without the addition of auxin. In the work with stem mustard, it was demonstrated that the application of GA_3 exhibited a profound effect on stem length, which was over three times longer after GA_3 application than after BA treatment, and that the stem diameter of microcuttings was significantly increased by BA in the absence of NAA [18,46]. In addition, it was reported that the application of BA to tulip stem substantially stimulated the thickening of all the internodes [54]. Presumably, aborted stem growth induced by the presence of BA and TIBA in the medium containing 9% (or 6%) sucrose could be the first step leading to stem swelling in kohlrabi plants grown *in vitro*.

Beside stem growth abortion, the application of PGRs induced leaf desiccation in kohlrabi plantlets, with almost 80% of plantlets affected when grown on C9 medium. Leaf desiccation in kohlrabi plantlets might be one of the factors that inhibited the priming of stem swelling signals. It is well known that mobile signals are primed in the source leaf upon exposure to inductive conditions. In Arabidopsis, FT is mobilized to the shoot apical meristem to activate flowering, whereas in potato, StSP6A and StBEL5 are mobilized to the underground stolon tip, the site of tuber formation [55]. Loss of stem coloration could also be observed in a certain number of plantlets after five weeks of culturing on all treatments, apart from the control and the combination of 6% and 9% sucrose with PGRs, which affected a larger number of plantlets. The pigmentation pattern observed in plants occurs because of the spatial distribution and accumulation of colored compounds, and may also be associated with structural changes to the tissue [56]; thus, the discolored pattern in kohlrabi stem could be an indicator of structural changes as well.

The use of PGRs in our *in vitro* experiments apparently did not induce stem thickening in kohlrabi, but stimulated callus formation and *de novo* organogenesis. The callus was developed on the kohlrabi stem after all applied treatments except in the control, and the strongest impact was noted for media with PGRs. This trend was also observed for shoot regeneration via *de novo* organogenesis that was only documented in plantlets grown on media enriched with PGRs. This is in accordance with our earlier published results on the successful induction of *de novo* shoot

organogenesis in kohlrabi hypocotyl explants and intact seedlings that were cultivated on media supplemented with different types of cytokinin, with BA and TDZ being the most efficient [30]. In the present study, the best response in terms of shoot regeneration was observed in C3 plantlets that were treated with 1 mg/L BA, 1 mg/L TIBA and 2 mg/L GA_3 in the medium containing 3% sucrose.

Carbohydrates serve as a carbon source to maintain the carbon supply as well as to maintain the osmotic potential of cells. Sucrose is widely used in plant tissue culture due to its very favorable effect on growth and its relatively low cost. There are also reports of the use of other sugars as suitable carbon sources for *in vitro* culture of different plants [7,8]. Glucose and fructose support growth of some plant tissues [57]; however, in our study, adding 1% xylose and fructose to the growth medium with 9% sucrose negatively affected kohlrabi plantlet height.

Mannitol is considered to be a compatible solute that can accumulate to high concentrations in plant cells but does not interfere with cell processes [58]. However, it may also act as a scavenger of activated oxygen species, which can damage plant cells [59]. Supplementing nutrient media with mannitol revealed that increasing concentrations of this sugar alcohol do not exert similar effects as other applied sugars. Mannitol induced osmotic stress and negatively affected kohlrabi seedling growth, indicating that the effects of xylose, fructose and sucrose are caused by alterations in osmotic potential. Loss of stem coloration, another altered feature of kohlrabi observed in this study, also appeared on M and S media.

The original purple kohlrabi cultivar used in this study is known for its anthocyanin accumulation in the leaf petiole and stem [60], with green as the normal color of leaves. Mannitol and high concentrations of sucrose in combination with glucose, xylose and fructose initiated changes in leaf color to dark blue-green, pointing to elevated synthesis and accumulation of anthocyanin as a stress response, which is consistent with our previous research. An earlier study demonstrated higher anthocyanin accumulation in cell suspension cultures of poplar after subjection to osmotic stress with glucose and mannitol [61]. Different carbohydrates have different effects on the biosynthesis of anthocyanin in different species, and sucrose is the main carbohydrate stimulating anthocyanin coloration [62]. A high concentration of sucrose has been shown to increase anthocyanin production in *Pistacia chinensis* leaves [63]. As a signaling molecule [14], sugar can induce the expression of genes in the anthocyanin biosynthesis pathway and promote the accumulation of anthocyanin [64].

Kohlrabi plantlets cultured on S, B and C, as well as M6 and M9 types of media, displayed changes in root appearance. High concentrations of glucose were shown to shorten the root meristematic zone in a dose-dependent manner [65]. Glucose reduces the size of the primary root meristem by increasing the rate of meristematic cell differentiation and by positively regulating the exit of cells from the primary root meristem into the elongation and differentiation zones. These phenotypic changes correlated with a reduction in auxin level in the roots. Deficiency in auxin transport protein BIG was shown to significantly sensitize the sugar-induced anthocyanin accumulation and sugar-inhibited primary root growth [66].

CONCLUSIONS

After comparing all media used in this study, it can be concluded that B6 and B9 media are the most potent media for further analyses of kohlrabi swelling considering their effects as follows: impairment of root formation, reduction in plantlet height, the lowest percentage of desiccated leaves, and the highest number of plantlets undergoing stem discoloration. Our results showed no tuber development after any of the *in vitro* treatments. However, the applied sugars altered a

variety of morphological aspects of kohlrabi growth and development in comparison to matching controls, while the use of PGRs stimulated callus formation and *de novo* organogenesis. Further investigation of the underlying mechanisms is necessary for a better understanding of stem swelling in kohlrabi and the role of carbon source type, plant hormones and/or other factors in this process.

Funding: This study was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia. Contract No. 451-03-68/2020-14/200007

Author contributions: SN and TĆ designed and wrote the manuscript, TĆ, JS, NG carried out the experiments, MR and AC participated in the design of the figures and graphs, and performed the statistical analyses, BV critically revised the manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1. Kohlrabi plantlets cultivated *in vitro* for 5 weeks on different growth media containing different concentrations of sucrose in combination with plant growth regulators **A** – Control plantlets grown on K3 medium (3% sucrose). **B** – Plantlets grown on M9 (9% mannitol) medium showing pronounced leaf desiccation. **C** – Dark blue-green leaves in plantlets grown on S2 (9% sucrose, 1% glucose + 1% xylose) medium differ from normal, green leaves in control plantlets. **D** – Loss of color in plantlet stems grown on B9 (9% sucrose + 1 mg/L BA + 1 mg/L TIBA) medium. **E** – Shoot regeneration in plantlet grown on B3 (3% sucrose + 1 mg/L BA + 1 mg/L TIBA) medium. **F** – Shoot regeneration on C3 (3% sucrose + 1 mg/L BA + 1 mg/L TIBA + 2 mg/L GA_3) medium.

Fig. 2. Average height of kohlrabi plantlets measured after 5 weeks of *in vitro* culturing on growth media containing different concentrations of sucrose combined with distinct growth regulators. Data are the mean±SE of three independent experiments with 10 replicates each. Means marked with the same letter were not significantly different according to Fisher's least significant difference (LSD) test, $P \leq 0.05$.

Fig. 3. Average height of kohlrabi plantlets measured after 5 weeks of *in vitro* culturing on growth media containing (**A**) 9% of sucrose combined with 1% of glucose, xylose and fructose, or (**B**) different concentrations of mannitol (3, 6, 9%), in comparison to control plantlets grown on media with 9% or 3% sucrose, respectively. Data are the mean±SE of three independent experiments with 10 replicates each. Means marked with the same letter were not significantly different according to Fisher's least significant difference (LSD) test, $P \leq 0.05$.

Fig. 4. Effects of different types of growth media on development of kohlrabi plantlets after five weeks of *in vitro* culturing. **A** – Percentage of plantlets with leaf desiccation. **B** – Percentage of plantlets with discolored stem. Data are the mean±SE of three independent experiments. Means marked with the same letter were not significantly different according to Fisher's least significant difference (LSD) test, $P \leq 0.05$..

Fig. 5. Percentage of kohlrabi plantlets with discolored stem recorded after 5 weeks of *in vitro* culturing on growth media containing (**A**) 9% sucrose combined with 1% glucose, xylose and fructose, or (**B**) different concentrations of mannitol (3, 6, 9%), in comparison to control plantlets grown on media with 9% or 3% sucrose, respectively. Data are the mean±SE of three independent experiments. Means marked with the same letter were not significantly different according to Fisher's least significant difference (LSD) test, $P \leq 0.05$.

Fig. 6. Induction of callus and subsequent indirect organogenesis in kohlrabi plantlets after five weeks of *in vitro* culturing on different growth media containing various concentrations of sucrose in combination with plant growth regulators. **A** – Percentage of plantlets forming the callus at the base of the stem plantlet. **B** – Percentage of plantlets with shoot regeneration. Data are the mean±SE of three independent experiments. Means marked with the same letter were not significantly different according to Fisher's least significant difference (LSD) test, $P \leq 0.05$.

Supplementary Material

The Supplementary Material is available at:
http://serbiosoc.org.rs/NewUploads/Uploads/Cosic%20et%20al_5530_Supplementary%20Material.pdf

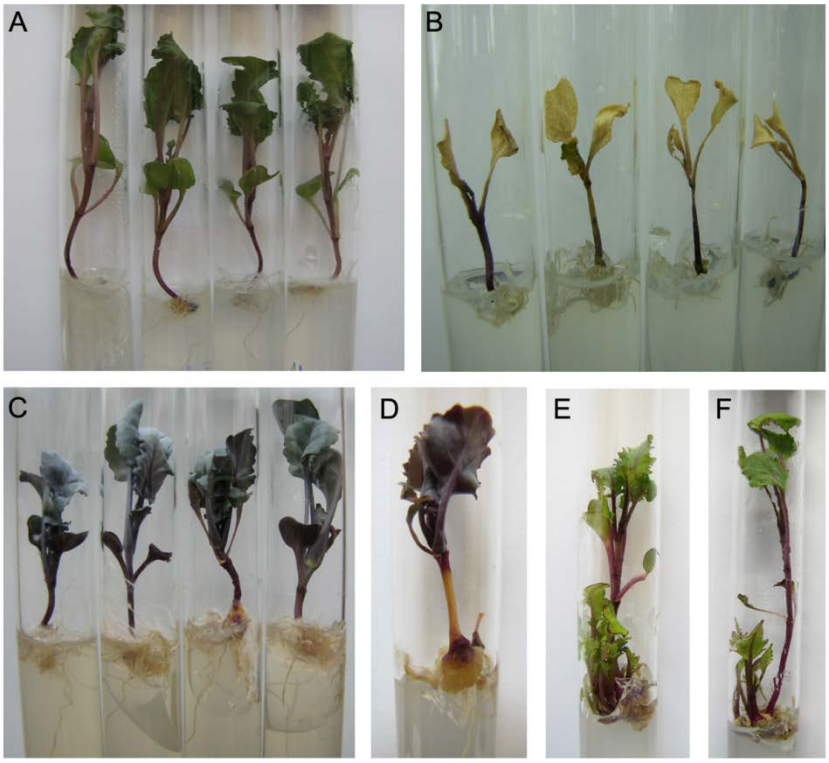

Fig. 1.

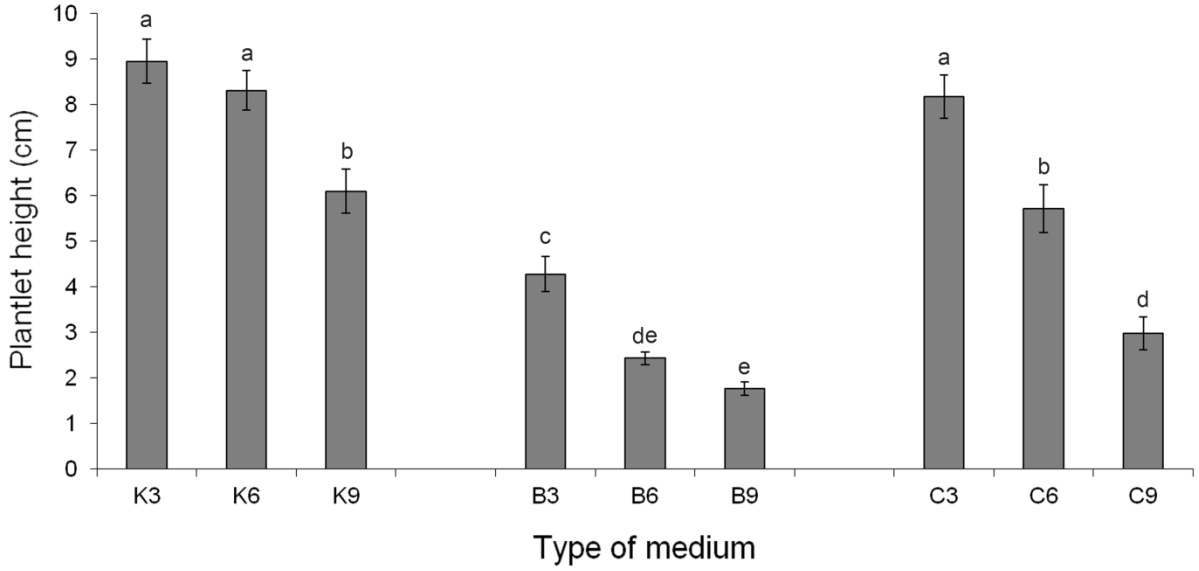
Plantlet height (cm)
10
9
8
7
6
5
4
3
2
1
0
a
a
b
c
de
e
a
b
d
K3
K6
K9
B3
B6
B9
C3
C6
C9
Type of medium

Fig. 2.

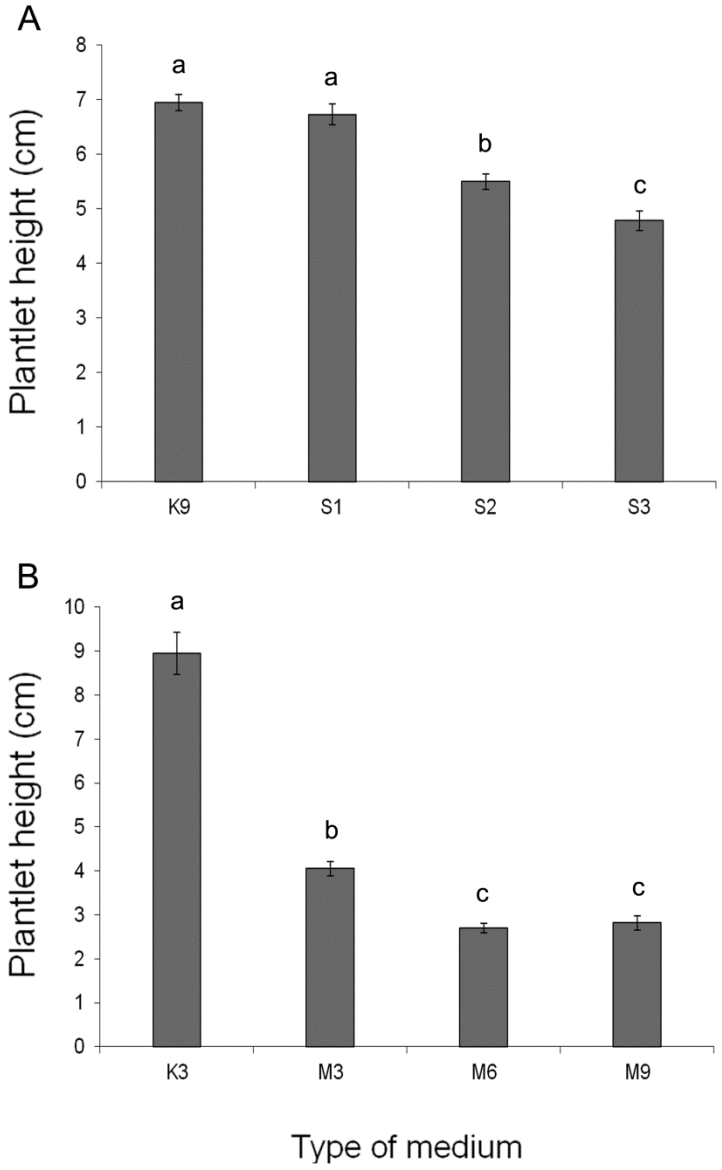
A
Plantlet height (cm)
a
a
b
c
K9
S1
S2
S3
B
Plantlet height (cm)
a
b
c
c
K3
M3
M6
M9
Type of medium

Fig. 3.

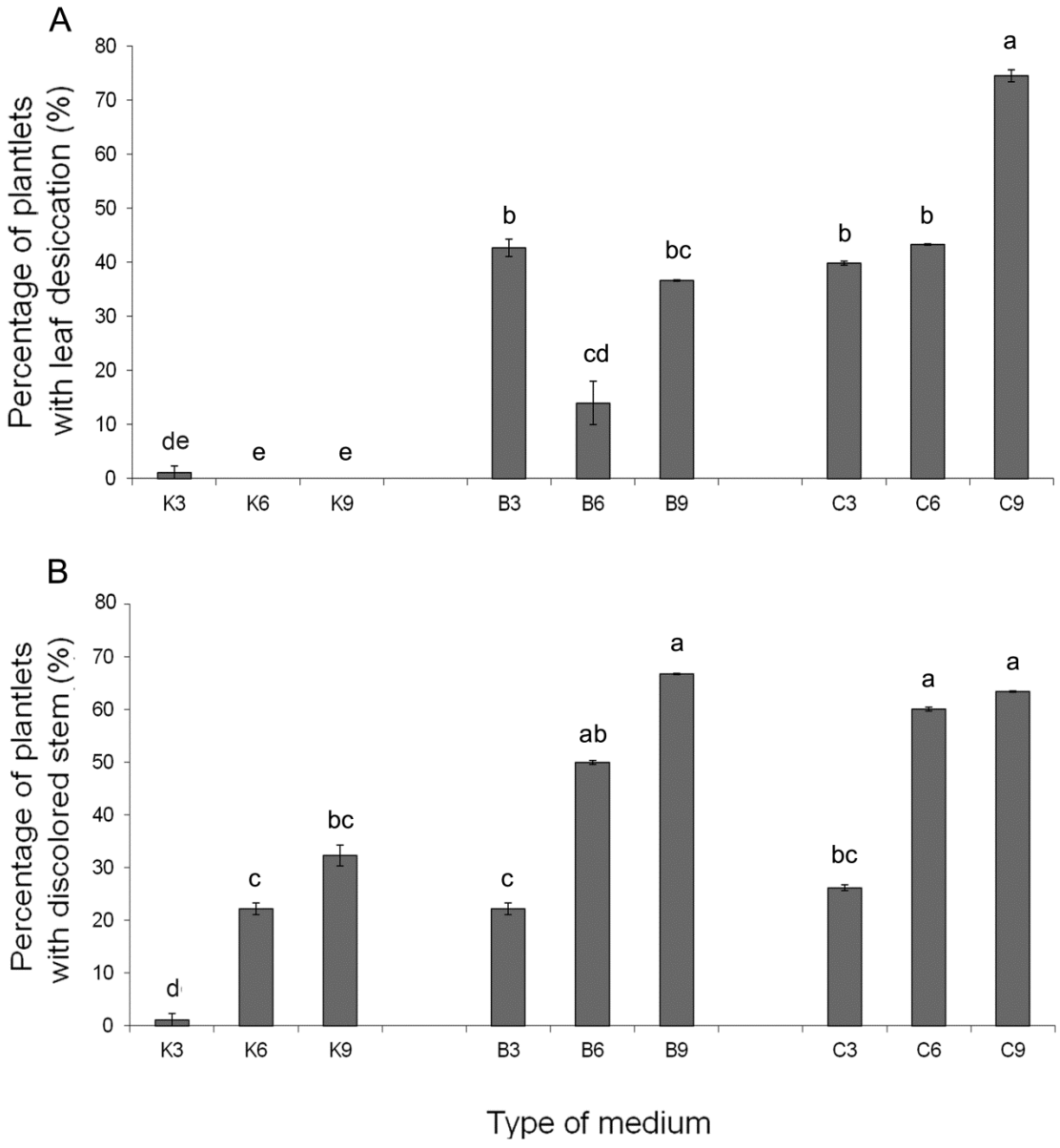
A
Percentage of plantlets with leaf desiccation (%)
80
70
60
50
40
30
20
10
0
de
e
e
b
cd
bc
b
b
a
K3
K6
K9
B3
B6
B9
C3
C6
C9
B
Percentage of plantlets with discolored stem (%)
80
70
60
50
40
30
20
10
0
d
c
bc
c
ab
a
bc
a
a
K3
K6
K9
B3
B6
B9
C3
C6
C9
Type of medium

Fig. 4.

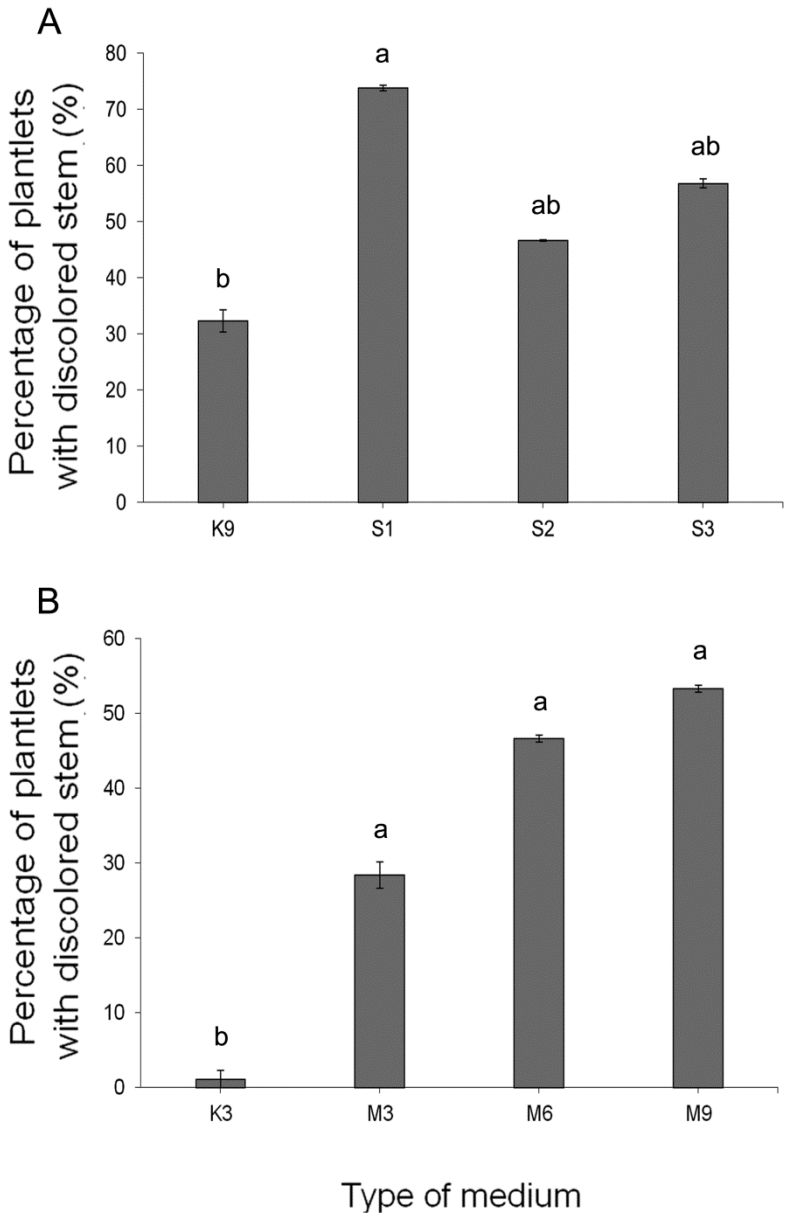
A
Percentage of plantlets with discolored stem (%)
80
70
60
50
40
30
20
10
0
a
ab
ab
b
K9
S1
S2
S3
B
Percentage of plantlets with discolored stem (%)
60
50
40
30
20
10
0
a
a
a
b
K3
M3
M6
M9
Type of medium

Fig. 5.

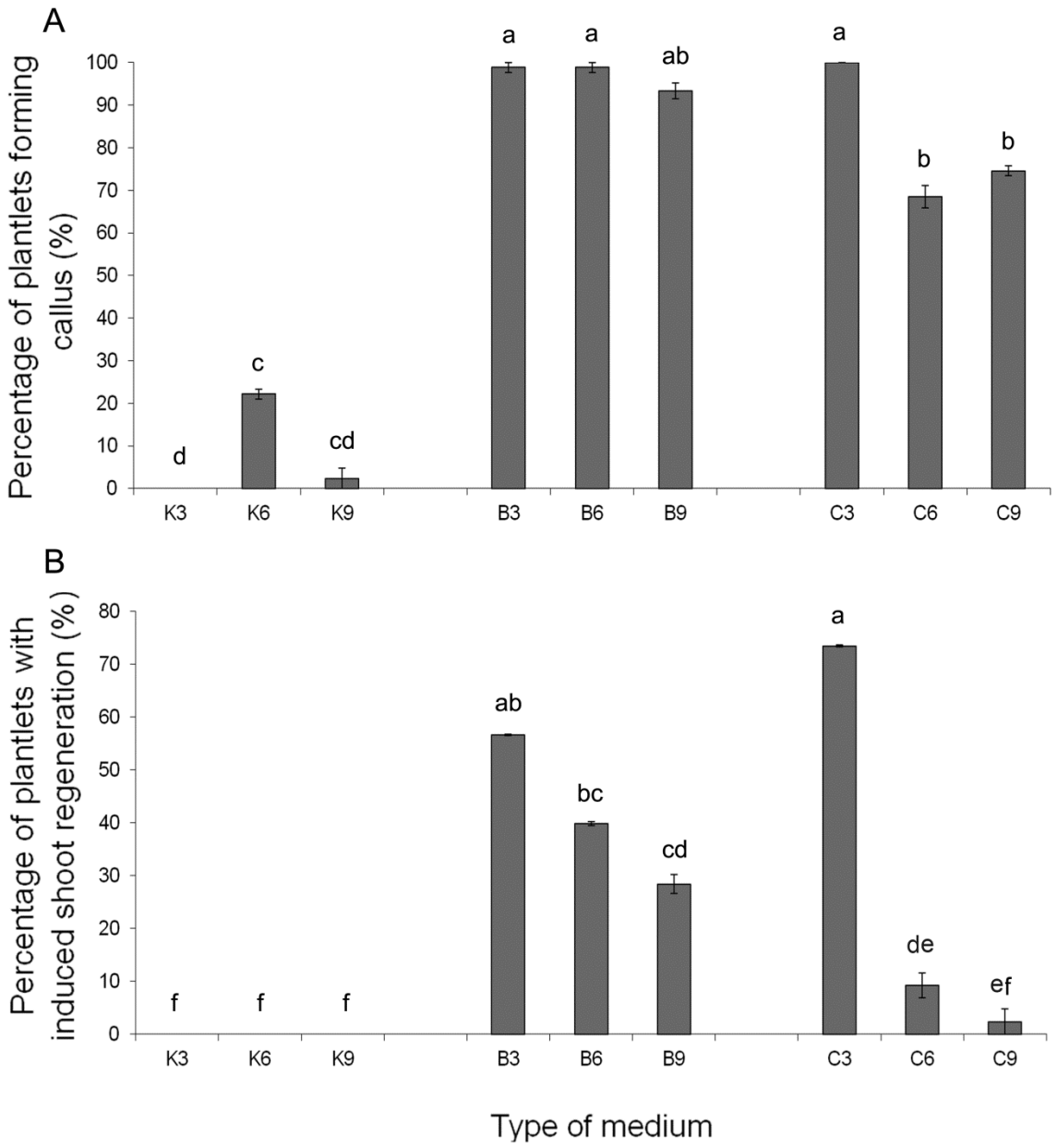
A
Percentage of plantlets forming callus (%)
100
90
80
70
60
50
40
30
20
10
0
d
c
cd
a
a
ab
a
b
b
K3
K6
K9
B3
B6
B9
C3
C6
C9
B
Percentage of plantlets with induced shoot regeneration (%)
80
70
60
50
40
30
20
10
0
f
f
f
ab
bc
cd
a
de
ef
K3
K6
K9
B3
B6
B9
C3
C6
C9
Type of medium

Fig. 6.