New methods for preventing the unwanted callusogenesis at sugar beet 
(Beta vulgaris var. Saccharifera)

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Abstract

Callusogenesis, an undesirable phenomenon and frequently encountered in sugar beet (Beta vulgaris var. Saccharifera) vitrocultures, was overcome by replacing bidistilled water - used as a solvent for all hydrosoluble components - in the culture media, with Pi water or deuterium (D) depleted water (DDW) (with 25 ppm D), even in the presence of 6-Benzylaminopurine (BA) in 2.5 mg/l concentration. The presence of the DDW in this culture medium has determined an 100% inhibitory effect of the callusogenesis, resulting in regeneration of vigorous and healthy plantlets; Pi water (PiW) also reduced callusogenesis at a rate of 10 times against the control - but compared with DDW, its inhibitory efficacy was lower.

Keywords: Beta, callusogenesis, Pi water, deuterium depleted water

Introduction

Sugar beet (Beta vulgaris var. Saccharifera) represents one of the most important crops [1, 2], being used for sugar and ethanol production. The best cultivars of B. vulgaris are cloned by micropropagation procedures [3]. In such practices, the callusogenesis emergence is quite frequent, which decreases efficiency in the cloning of selected genotype endowed with resistance to rhizomania. In micropropagation industry, although there are numerous plant material losses, the economic damage following the appearance of callusogenesis phenomenon exists [4] - yet solutions to stop this could not be found.

When the callus is young it has a particular morphoanatomical structure, composed of proliferative and undifferentiated cells in parenchyma tissues. At the periphery of a normal callus, there is a primary meristem, and organogenic or embryogenic centers, depending on the nature of growth regulators present in the culture medium, and the type of tissues, which served as explants for in vitro culture initiation [5]. Callus cells are characterized by low activity of enzymes involved in synthesis of lignin precursors and enzymes responsible for polymerization [6]; in olive callus was found that the level of chlorophyll b is higher than that of chlorophyll a [7].

The callus development is possible through the proliferation of cells. It is the result of a lawless, anarchic proliferation, sometimes correlated with physiological disorders like hyperhydricity, and the cells differentiate into vascular elements, without the tissue organization [8, 9]. Hormonal manipulation of this normal callus display allows the expression of the organogenic potential through shootlets and rootletts, but sometimes the presence of callus can have harmful, unintended effects.

In our experiments we analyzed the effect of Pi water (PiW) and deuterium depleted water (DDW) respectively, on sugar beet in vitro regenerated plantlet callusogenesis, following some reports which demonstrated that DDW influenced the growth rate of animal
tumor cells [10]. In mice, using DDW instead of natural water, a significant decrease of cancer cells multiplication was recorded. Somlyai and his colleagues [11] found that a reduction in deuterium content of the drinking water for animals influenced the cell metabolism until a blocking up in the development and even the disappearance of tumor cells was observed. In these authors opinion the cells are able to regulate deuterium (D)/hydrogen (H) ratio and the changes in this rapport can trigger certain molecular mechanisms having a key role in cell cycle regulation [12]. The authors suppose that the concomitant increase of D/H ratio is the real trigger for the cells to enter in the S phase of division [13]. The decreases of D level can intervene in the signal transduction pathways thus leading to tumor regression. In the case of transplantable tumors, low-deuterium water treatment resulted in significant inhibitory effect on volume of all tumor patterns concerned, delaying the nodule formation at transplantation site.

On the other hand, in plants, deuterated water was used as tracer to characterize whole-tree water transport and storage properties in individual trees belonging to the coniferous species [14] and in five tropical tree species and a bamboo species [15]. The deuterated water tracing method appears suitable for answering some questions, such as relative differences in water use among trees, water redistribution among neighbours and internal water transport and storage processes in plants [15]. Also, the effects of combined soil physical stresses of compaction and drought on the production of fully hydrated mucilage and root border cells (RBCs) in maize was studied using deuterated water method [16].

*Chlorella protothecoides* (a lutein-producing microalga) was grown aerobically in a mineral medium prepared with 70% (v/v) deuterated water [17] and the rapidly growing microalga had much higher levels (58%) of deuterated lutein relative to previously reported (9–15%) natural sources of lutein.

**Materials and methods**

Dragged seeds of sugar beet were disinfected - for 15 minutes - in sodium hypochlorite solution, (40% concentration), to which were added a few drops of Tween 20, followed by rinsing in five bath of sterile water to remove traces of chlorine; then, the seeds were inoculated on aseptic mineral basic medium (MB) Murashige - Skoog (MS) [18] at half (½) concentration, solidified with 7 g/l agar-agar, without growth regulators, prepared with bidistilled water (Table 1). The medium pH was adjusted to 5.7.

In the 30-th day after the *in vitro* initiation of the seed germination experiment the sugar beet plantlets developed from the zygotic embryos (at a rate of 89%) generally had a rootlet with an average size per whole lot of 2.4 cm, a stemlet with an average length of 5.5 cm, on which were inserted the two cotyledons, and two leaflets in the top of the plantlets. Uninodal, apical minicuttings of 2-3 mm size were detached from the plantlets and were inoculated on MB-MS media, prepared with different types of water, respectively bidistilled water (DW), Pi water (PiW) or deuterium depleted water (DDW), the last one with a content of 25 ppm D, with or without the addition of BA (6-Benzylaminopurine), according to the protocol from Table 1.

After 30 days of *in vitro* culture of sugar beet apices the morphogenesis, structural and ultrastructural aspects of calli developed at the base of minicutting explants - which were cultivated on culture media prepared with DW or with PiW (only the batches which presented callus) - were studied. Callus fragments were taken and fixed and were processed, according to the transmission electron microscopy specific techniques [19].

The callus fragments fixation was made by 2.7% glutaraldehyde solution, for one hour, after that the fragments were post-fixed in 2% osmic acid solution, and then were dehydrated
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in increasing concentrations of acetone baths; later, the vegetal samples were included in EPONE 812. The sectioning of sample blocks was performed with a Leica UC6 microtome, and the contrasting has been made with uranyl acetate and lead citrate solutions. Preparations were examined at a transmission electron microscope (Jeol JEM1010), and images were photographed with a brand digital camera Mega View III CCD. The photographs were processed through Corel Photo Paint 12.

The callus subculture was performed by detaching callus fragments from the base of the shoots developed on the media prepared according to V00 and V1 experimental variants, and transferring them on the medium with 2 mg/l 2.4-D (Table 1).

**Table 1.** The experimental protocol for *in vitro* culture of sugar beet (*Beta vulgaris* var. Saccharifera) seeds, caulinar explants and callus subcultures.

<table>
<thead>
<tr>
<th>First culture (initiation seeds) - 30 days -</th>
<th>Culture medium in the first subculture - 30 days -</th>
<th>Callus subculture</th>
<th>Culture conditions</th>
<th>Kind of biometrizations made in the final of first subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td>solidified mineral basic medium MB – MS ½, without growth regulators, prepared with DW</td>
<td>V00 - MB – MS + 2.5 mg/l BA prepared with DW (control)</td>
<td>MB – MS + 2.4 - D, 2 mg/l, prepared with DW</td>
<td>- 23°C ± 2°C in lightness period; - 20°C ± 2°C, in darkness period; - 1700 lx; - 16/24 h photoperiod.</td>
<td>No. of rootlets/inoculum L. of principal rootlets No. of total leaflets/inoculum No. of green leaflets/inoculum No. of necrosed leaflets/inoculum No. of rosettes/inoculum L. of petioles L. of foliar limb L. of stemlets L. of plantlets Callusogenesis degree/experimental variant Structural aspects of callus Ultrastructural aspects of callus</td>
</tr>
<tr>
<td>V0 - MB – MS without growth regulators, prepared with DW</td>
<td>V1 - MB – MS + 2.5 mg/l BA prepared with PiW</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>V2 - MB – MS + 2.5 mg/l BA prepared with DDW</td>
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</table>

*Note: 2.4 - D – (2.4 – dichlorophenoxy)acetic acid; BA – 6-Benzylaminopurine; DW – bidistilled water; DDW – deuterium depleted water; L – length; MB – basal medium; MS – Murashige – Skoog medium [18]; no – number; PiW – Pi water.*

**Results**

1. **Evolution of apical uninodal minicuttings on media prepared with different types of water**

After 30 days from the first subculture, the growth indices of plantlets regenerated from uninodal, apical minicuttings of sugar beet, and the values recorded in the variants with the BA addition in the culture medium prepared with different water types (variants V0, V1 and V2) have been reported to the average calculated from control group (V00) (dates considered as 100%), all with the addition of 2.5 mg/l BA, but prepared with DW are represented as histograms inserted in Figure 1.

Regarding *in vitro* rhizogenesis, only to regenerated plantlets on MB - MS + 2.5 mg/l BA prepared with DDW (V2) appeared two adventitious roots at the basal region of each stem, with the average length of 0.3 cm. This root genesis was possible because on the base of shoots callusogenesis was absent; this phenomenon was present in other experimental variants on the culture medium which contained BA, especially on those prepared with DW (V00) (Figure 2 A and B).
Caulogenesis, from the perspective of the leaflet total number was stimulated by the growing medium with the addition of BA and prepared with DW (V00) (Figure 1).

An inhibitory effect on new leaflets development on sugar beet shoots has been recorded in the presence of both water PiW (V1) or DDW (V2), the newly-regenerated shoots, on this culture media, marking minuses by 23%, comparatively with those which was raised on the control culture media (V00) (Figure 1).

Although the total leaflets number was higher in the control group, the most vigorous, intensely green and the largest number of green leaflets were reported on shoots regenerated on medium prepared with DDW (V2) (Figure 1 and Figure 2 B), which was the lot that we’re not necrosed leaflets (Figure 1 and Figure 2 B).

An advantage offered by the phytoinocula cultured on medium with PiW (V1) or DDW (V2) - compared to those regenerated on medium containing DW (V00) - was the development of rosettes, in which the leaflets, although they were fewer in number, grew normally arranged at node on 2 - 3 levels (Figure 1), situation which did not appear in the control group, where the intense callusogenesis affected the rosettes development (Figure 1). Hence, the appearance of several buds in calli resulted in an increase of the total leaflet number.

With regard to leaf size components, the longest foliar limbs, on average of 1.5 cm, were measured in shoots cultivated on medium with DDW (V2); the values were 50 % plus, compared with DW (V00) and the lowest values of foliar limbs were identified at leaflets regenerated on medium prepared with PiW (V1); in the last case the minuses was 50% comparatively to the control (Figure 1).

The leaflets on the shoots cultivated on the culture media without BA, and prepared with DW (V0) had petioles sizes similar to those grown on medium with 2.5 mg/l BA and prepared...
New methods for preventing the unwanted callusogenesis at sugar beet (*Beta vulgaris* var. Saccharifera) with DDW (V2), but the difference between them consists in petioles color and thickness, so that those of the DDW (V2) group were greener and more vigorous.

**Fig. 2.** Aspects of (*Beta vulgaris* var. Saccharifera) plantlets grown – for 30 days (A) and for 90 days (B) – from uninodal, apical minicuttings subcultured on MB – MS aseptic medium, according to the following experimental variants: V00 – MB - MS + 2.5 mg/l BA, prepared with distilled water; V0 – MB - MS without growth regulators, prepared with distilled water; V1 – MB - MS + 2.5 mg/l BA, prepared with Pi water; V2 – MB - MS + 2.5 mg/l BA, prepared with deuterium depleted water (25 ppm D) {BA – 6-Benzylaminopurine; DW – bidistilled water; DDW – deuterium depleted water; MB – basal medium; MS – Murashige – Skoog [18] medium; PiW – Pi water; bars means 1 cm}.

The leaflets number was superior for shoots grown on the control medium prepared with DW (V0) but not the same thing was observed in the case of stemlet lengths in these groups. The smaller size was identified in the control group, because many recorded leaflets were not arranged in rosettes, in superimposed verticiles, as was the case of plantlets cultivated on the variants in which DW was replaced with PiW (V1) and especially those grown on media with DDW (V2), but presented more newly-formed shoots at the callus level, so the size of the stemlet were 200% higher, in the case of V1 variant, respectively 260%, to lot noted with V2 (Figure 1).

Plantlet length coincided with the stemlet lengths in shoots which had the rhizogenesis hampered by the callus emergence (V1), but it was different and highest (showing 320% plus, as seen in Figure 1) in plantlets regenerated on medium prepared with DDW (V2).

2. **Regenerating callus at the base of uninodal caulinar apical minicuttings**

In this experiment, after 30 days of vitroculture, only plantlets regenerated from caulinar apices of sugar beet, cultivated on medium without growth regulators, respectively, without BA (V0) and that prepared with DDW (V2), even in presence of BA (Figure 1) have not presented callusogenesis.

At the basal level of shoots grown on medium prepared with DW plus BA 2.5 mg/l (V00), compact masses of callus were formed. In this variant, which we considered the reference, this callus size was 3 cm in length and the percentage of necrosis was 90% and the consistency was hard. In this group, the callus presence at the shoot basis stopped the rhizogenesis, even in an advanced growth stage. Also on leaflets which were in direct contact with the culture medium, it was the dedifferentiation and callus formation.

Compared with control variant (callus formed on the basis of phytoinocula grown on medium MB-MS, with 2.5 mg/l BA and prepared with bidistilled water), replacement of DW in the culture media with PiW (V1) has an inhibitory effect on callusogenesis. The basis of shoots which were grown on such medium (even in the presence of 2.5 m/l BA) currently has callus, with was 10 times lower than the control size (Figure 1). On culture medium prepared with PiW (V1) the plantlet leaflets that came into contact with the culture has entered a dedifferentiation process, having newly-formed callus all over them.
Culture medium which was prepared with DDW (V2) had an inhibitory effect on callusogenesis, therefore in the plantlets grown on such a medium the presence of callus was not recorded, which allowed (after 90 days of vitroculture) formation of vigorous adventitious rootlets organized in a well-developed radicular system at the first node of stemlets, which was sticking in the culture medium (Figure 2 B).

If the initiation of callus in sugar beet (Beta vulgaris var. Saccharifera) was induced on culture medium MS-MB (1962) with 2.5 mg/l BA addition, the subcultivation of it on medium MB - MS, with 2.4-D, in concentration of 2 mg/l (without BA addition), prepared with bidistilled water, after 30 days of this subcultivation, the callus was 10% necrotic and 90% hyperhydric, with friable consistency. The culture media with 2 mg/l 2.4-D have produced only vitrified callus (Figure 3).

Fig. 3. Aspects of sugar beet (Beta vulgaris var. Saccharifera) hyperhydric callus at 30 days from their subcultivation on MB – MS medium with 2 mg/l 2.4-D {2.4 - D – (2.4 – dichlorophenoxy) acetic acid; MB – basal medium; MS – Murashige – Skoog [18] medium}. 

A. Normal callus of sugar beet, generally that developed on culture media prepared with water Pi (V1) - presented parenchyma cells with regular shapes and sizes (Figure 4 A - B) and

Fig. 4. Optical microscopy aspects observed in longitudinal sections through sugar beet (Beta vulgaris var. Saccharifera) normal callus provided from culture medium prepared with Pi water (V1) (A and B) and hyperhydric callus formed on medium prepared with distilled water (V00) (C and D), regenerated at the base of shoot, during 30 day from subcultivation (c.f.- corpuscular formations; i.sp. – intercellular space; L – lacuna; mx – myxoplasm; N – nucleus; V – vacuole; X - xylem) (A – 100X and B – 200X; C – 100X; D – 400X).
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5, A - B), with small intercellular spaces, and with rare elements of xylem in its depth; the normal callus presented, too, parietal cell cytoplasm and a large vacuole, with an integral tonoplast (Figure 5 A and B).

![Ultrastructural aspects observed in sugar beet (*Beta vulgaris* var. Saccharifera) normal callus provided from culture medium prepared with Pi water (V1) (A and B) and hyperhydric callus formed on medium prepared with distilled water (V00) (C and D), regenerated at base of shoot, during 30 day from subcultivation (cyt - cytoplasm; c.f. – corpuscular formations; c.w. – cell wall; chl – chloroplasts; i.sp. – intercellular space; L – lacuna; l.d. – lignin deposits; mx – myxoplasm; V – vacuole; N – nucleus; n – nucleon) (bars means: A – 10 µm; B – 2µm; C – 10 µm; D – 5 µm).](image)

*Hyperhydric* sugar beet callus formed during 30 days at the base of shoots grown in culture medium with the addition of BA and prepared with DW (V00) consisted in a mass of deformed cells (Figure 4 C and D), especially those from outward area, with large intercellular spaces, transformed into real gaps (Figure 4 D and Figure 5 C and D); in many cells was observed the dismantling of tonoplast and vacuolar juice mix with cytoplasm, forming a myxoplasm, which shows a permanent alteration of cell structure. These aspects confirm ultrastructural disintegration and malfunctioning status of hyperhydric callus, which presents no differentiation and morphogenetic capacity. Also as a feature of hyperhydric callus of beet it is to mention the frequent appearance in vacuolar juice of corpuscular formations, electron dense, of unknown origin (Figure 4 D and 5 C). In hyperhydric callus, often, appear cells which undergo necrosis (Figure 5 D).

**Discussions**

Comparing the parameters registered in the control group - phytoinocula grown on media prepared with bidistilled water – with the sugar beet shoots regenerated from caulinar, uninodal apices, grown on culture medium Murashige – Skoog [18], with 2.5 mg/l 6-benzylaminopurine addition, in which bidistilled water was replaced by Pi water or deuterium depleted water (D having 25 ppm) a stimulation of stemlet elongation was observed, while
the rhizogenesis was present only at the shoots basis which were cultivated on medium prepared with deuterium depleted water, with 25 ppm deuterium concentration.

Culture medium prepared with deuterium (D) depleted water - with D 25 ppm - in presence of BA, expressed an inhibitory effect of plantlet callusogenesis and determined the regeneration of vigorous and healthy plantlets.

Pi water manifested the same inhibitory effect on the phenomenon of callusogenesis, though at a lower level of efficacy compared with deuterium depleted water, but with a much better effect compared with distilled water.

Other results regarding the effect of DDW or PiW on callusogenesis inhibition are not presented in the literature to our knowledge. We previously studied the positive effect of both waters in hyperhydricity prevention [20] and annihilation [21, 22]. In some transgenic Coffea spp., the glufosinate was reported to efficiently inhibit the growth of leaf callus and callus suspensions without inducing necrosis [23].

There is a species-specific inhibitory effect of allelopathy on cultured cell and callus growth [24]. The soybean (Glycine max Merr.) callus was cultured with rice (Oryza sativa L.) callus in the same culture bottle, and allelopathic evidence of growth inhibition on the former callus was observed. The allelopathic effect of inhibition on soybean callus was non-specific with respect to rice cultivars.

In the case of Beta vulgaris callus used in transformation experiments with selectable markers, the antibiotics geneticin, gentamycin, hygromycin, kanamycin and phleomycin were screened utilizing 'Betaseed 4587' [25]. Callus growth was inhibited by levels of 50 µg/ml geneticin, 150 µg/ml gentamycin, 10 µg/ml hygromycin, 150 µg/ml kanamycin and 20 µg/ml phleomycin. The results indicate that the concentrations of antibiotics used to inhibit callus induction was sufficient.

In our experiment we obtained callus only at the basis of sugar beet (Beta vulgaris var. Saccharifera) minicuttings which were grown on culture medium with 2.5 mg/l 6-benzylaminopurine, prepared with bidistilled water. Replacement of bidistilled water from the culture medium of sugar beet (Beta vulgaris var. Saccharifera), with Pi water, especially with deuterium depleted water (with 25 ppm D) proved to be a very efficient method, cheap and handy, in preventing the occurrence of the callusogenesis phenomenon in these vitrocultures.

The callus regenerated on the base of shoots on culture medium prepared with Pi water has normal ultrastructural aspect (with xylem representation), while that formed on medium prepared with distilled water was hyperhydric. After Bader and his collaborators [26, 27], Forsytia cells - from normal callus cultures - had less starch, in cytoplasm could see Golgian elements could be seen, endoplasmic reticulum and frequent multivesicular body; the mitochondria showed slightly swollen crista and a variety of corpuscular aggregates and filamentous spheroglobule were commonly found in vacuolar juice. The authors noted that, although ultrastructural picture was found to be a fairly complex one, showing necrotic callus cells – the callus presented rhizogenetic capacity.

The hyperhydricity phenomena frequently present in sugar beet callus was also reported [5]. In these cases callus had a luxuriate growth, was friable, lacking chlorophyll, and without morphogenic capacities. In hyperhydric callus culture of cactus, peroxidase and esterase activity was higher [28]. Eboe–Nil and Al-Sabah - cited by Cachiţă and collaborators [29] - showed that hyperhydric callus cultures of Rhanterium epapposum (an evergreen shrub in desert areas) can be controlled by adding in the growing medium of 1-30 mg/l silver nitrate, compound which is an inhibitor of endogenous ethylene production. The addition of certain compounds which have been observed to control callusogenesis, as reported by other authors [10, 29] supports the findings of this study.
References


