EFFICIENT INITIATION OF *IN VITRO* CULTURE AT WHEAT

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Abstract. Wheat is one of the most important crop plant species used for food and feed as well as in the bioethanol industry and therefore it was in the center of biotechnological research and it is still present. The main aim of this article is to investigate the efficiency of the Blidar type filter-paper bridges in initiating the wheat *in vitro* cultures for liquid culture media, in comparison with the conventional agarized culture media – solid culture media. In these experiments it were used modified Murashige-Skoog culture media (1962) (free of AIA and amino acids) supplemented or not with agar. The agarized culture media is used as a control and the liquid culture media is used for being provided with filter-paper bridges designated to maintaining the inocula at the upper level of the liquid culture media. The inocula consisted in caryopsis of *Triticum aestivum* L. (hybrid Kiskun Gold - HU 142 238). Based on the results of these experiments it can be underlined that growth increases for the cultivated vitroplants on liquid culture media provided with filter-paper bridges compared with those conventianlly cultivated on an agarized culture media, as following 2.55% for fresh weight and 55.27% for hypocotyls length.

Keywords: *in vitro*, filter-paper bridge, initiation, wheat, plant biotechnology

INTRODUCTION

Wheat is one of the most important crop plant species used food and feed as well as in the bioethanol industry which is considered today to be as the fuel of the future [23]. At the global level, in 1999, were produced 587.6 million tons of wheat for 213.3 million hectares and ten years later, the wheat production increased at 686.9 million tons for an increased cultivated area of 225.6 million hectares [16], underlying the growing tendency. This process is explained through the demographic explosion we are controling today and climate change policy, which means that there is an even more acute need of food and green fuel [36], which can be overcomed by increasing the cultivation efficiency of existing surfaces, respectively using sustainable bioeconomy [12, 27, 39], based on Environment Conservation and food biosecurity Factors in relationship to climate change [1] that make up today's new paradigm - eco-bioeconomics [11], or, in the worst case, using new surfaces for culture [48].

No matter the path which is to be followed for our future development, involving plant biotechnologies and genetic engineering is inevitable, because through them, in a short time can be achieved (months or years at most) improved new hybrids or varieties of more productive and resistant plants on the existing fields, or for new ones included in the agricultural circuit [13, 35], and on the other, shortening the period of *in vitro* cultivation [14, 17] implies a lower cost price, respectively a proportional decrease especially in electricity consumption needed to preserve optimal physical conditions in the rooms of vegetation. Therefore, making more cost-efficient the *in vitro* cultures of species either for conservation either as a tool for breeding process, is today one of the major concerns of scientists [38].

Modern biotechnology such as: tissue cultures, genetic engineering, and genetic transformation techniques, provide new opportunities to enhance the germplasm quality in crop plants [41]. A reliable shoot regeneration protocol is a prerequisite for efficient application of genetic transformation strategies [19]. Several regeneration protocols involving shoot organogenesis from callus [21, 30] and somatic embryogenesis from immature embryos [33, 34], mature embryos [8, 15, 37, 46], from scutella [5, 25], or in cell suspension [43] in wheat have been reported with varying successes. In wheat, were also reported results regarding the callus induction from caryopsis [18, 30], or immature embryos [6, 49], and indirect plant regeneration from callus [6, 8, 21, 42, 44, 45]. There were also highlighted optimum methods for cell suspension culture production originated from immature embryos [9], and also related to the resistance of the *in vitro* wheat plants for different unfavourable weather conditions [40]. Histological studies completed *in vitro* embryonic development [4] in wheat. Also, *in vitro* haploid production systems [22, 50], as well as *in vitro* mutation techniques [26] have been developed during time. All these studies were made *in vitro* culture conditions, which implied an initiation step of the *in vitro* cultures essential for any *in vitro* technique development.

So far, into the specialized literature there are few references regarding *in vitro* culture initiation on liquid culture media provided with filter-paper bridges, but none of them is referring to the advantages that this technique brings to initiate cultures in aseptic environment compared to the use of an agarized medium. Regarding wheat, it was demonstrated that both total and embryogenic callus were doubled and significantly a higher number of regenerants was obtained on liquid culture media provided with filter-paper bridges compared to the cultivation on solid culture medium [29]. For potato the proposed method for cultivating in liquid culture medium provided with filter-paper bridges has been successfully obtained the preservation for 3 years of microplantlets belonging to 360 cultivars, at 10°C and 2 Klux light (16 hours light/24 hours) in test tubes containing 5 ml Murashige and Skoog (MS) liquid culture medium. After that period these inocula were preserved through

cryopreservation, and finally, the average regeneration of all potato cultivars was about 40% [28]. Later the filter-paper bridge technique was used in liquid culture media for the regeneration of cell lines, such as for tobacco, which have undergone various tests to highlight the effect of 3,4-dihidroxybenzoic acid on root membrane potential, establishing that they have been preconditioned with this substance, indole-3-acetic acid activity on cell membrane which was altered, suggesting a specific reciprocal interaction [31]. In *Medicago sativa* it has also been achieved the start of seed cultures using liquid culture media provided with filter-paper bridges introduced into 25 ml test tubes containing 5 ml of water, in order to determine if genetic differences in winter hardiness between dormant and non-dormant alfalfa were retained by suspension cells derived from these contrasting cultivars and to determine the physiological and biochemical bases for differences in freezing tolerance of suspension cells [20]. The filter paper-bridge as a technique was also used *in vitro* to study the effects of saline and osmotic stresses on proline and sugar accumulation in *Populus euphratica*. At the end of the experiment it has been proved that *P. euphratica* displays tolerance for osmotic and saline stresses at the *in vitro* plantlet level and it is suggested that the accumulation of proline and total soluble sugars in leaves maybe related to osmotic and saline stress tolerance [47]. Also, in *Cicer arietinum*, to study the role of enzymes and identification of stage-specific proteins in developing somatic embryos, *in vitro* cultures were initiated using seeds that were placed on filter-paper bridges for liquid culture media, from the vitroplants, smearing the mature cotyledons [24].

In the present study it is described an efficient method to initiate *in vitro* cultures of wheat, using Whatman filter-paper bridges for liquid culture media.

MATERIALS AND METHODS

Plant material and growth condition. Uniform and healty mature caryopsis of winter weaht (*Triticum aestivum* L.) cultivar Kiskun Gold hybrid (HU 142 238) were selected for this experiment and kept under running tap water for 15 min. Then they were disinfected using solution of commercial bleach (ACE Automat – which contained about 5% sodium hypochlorite) for 10 min and rinsed with sterile distilled water, five times to remove traces of sodium hypochlorite. After that, the caryopsis were cultivated aseptically on a modified culture medium (aminoacids and hormone free) Murashige – Skoog (1962) [32] (MS) basal medium, either agarized (with 7 g/l agar-agar) – V_0T experimental variant (control), either liquid, inocula was supported at the surface using a Blidar type filter paper-bridge [10] (V_1T variant). The medium pH was adjusted to 5.7. The cultures were maintained in a growth chamber at 23±1 ^{0}C under a 16-h photoperiod (20 μM $m^{-2}s^{-1}$ PAR) by warm white fluorescent tubes.

Growth measurement. The observation and measurements were performed until the 21st day of *in vitro* culture, at an interval of 7 days. Were analyzed and compared biometric following parameters per plant: length of roots; length of coleoptiles; length of leafs; leafs number (excluding coleoptiles); fresh weight and dry weight of plant material.

Statistical analysis. The values registered at control group (V_0T) was considered the reference for each of the three experimental data when analysis were performed. The statistic significance of difference related to control variant was calculated by Student's T-test, for two tailed strings with unequal variances. Experiments were repeated twice, each time with three replicates with 20 caryopsis in each replication. All statistical analyses were performed with SPSS, v.16.0.

RESULTS

The use of filter-paper bridges proved to have beneficial effects on the growth of wheat *in vitro* plantlets, whereas both in terms of morphological and biometric and gravimetric parameters values, the achieved results were higher compared to the corresponding controls, as it can be proved further in this article.

In the case of caryopsis placed on filter-paper bridges, at the basement part of the small roots, the presence of a friable regenerative callus, extended for 3-4 mm length and 1 mm thickness (Fig. 2A) in *7 days* of starting experiments, was observed.

At this time of experiment, excepting the parameter values of *fresh biomass*, all other values for the studied parameters, were superior for medium variant on filter-paper bridge (V_1T), compared with those on the agarized culture medium (V_0T). The highest difference was observed for the *length coleoptile* (i.e. 24.14 mm in length, with 58.2% superior compared to the control) and the lowest for the amount of *dry weight* (i.e. 43.5 mg, 10.1% higher than the control). Regarding the *fresh weight* of the plant's biomass, the values marked at the control group (V_0T) were higher compared than the tested group (V_1T), but only with 2.2%, which means in absolute values a difference of only 3.6 mg / plant (Fig. 1A-F & 2A).

At *14 days* of *in vitro* culture, regarding the morphological aspect, it must be noted that in case of in vitro plantlets developed on the agarized substrate, an early calusogenesis phenomenon occurred. The friable regenerative callus, was extended starting with the base of the roots, but only for a length of 2-3 mm and 1 mm thickness. On contrary, in case of the new-developed *in vitro* plantlets on liquid culture medium with filter-paper bridges, the layer of callus was extended on a length of 4-5 mm from the roots, plus a length of about 1 mm from the coleoptiles. Also, in the case of V_1T variant it was noted that some of the roots become light green (Fig. 2B).

The above described characteristics for 7 days of cultivation, were amplified in terms of the observed parameters, compared to control. The highest

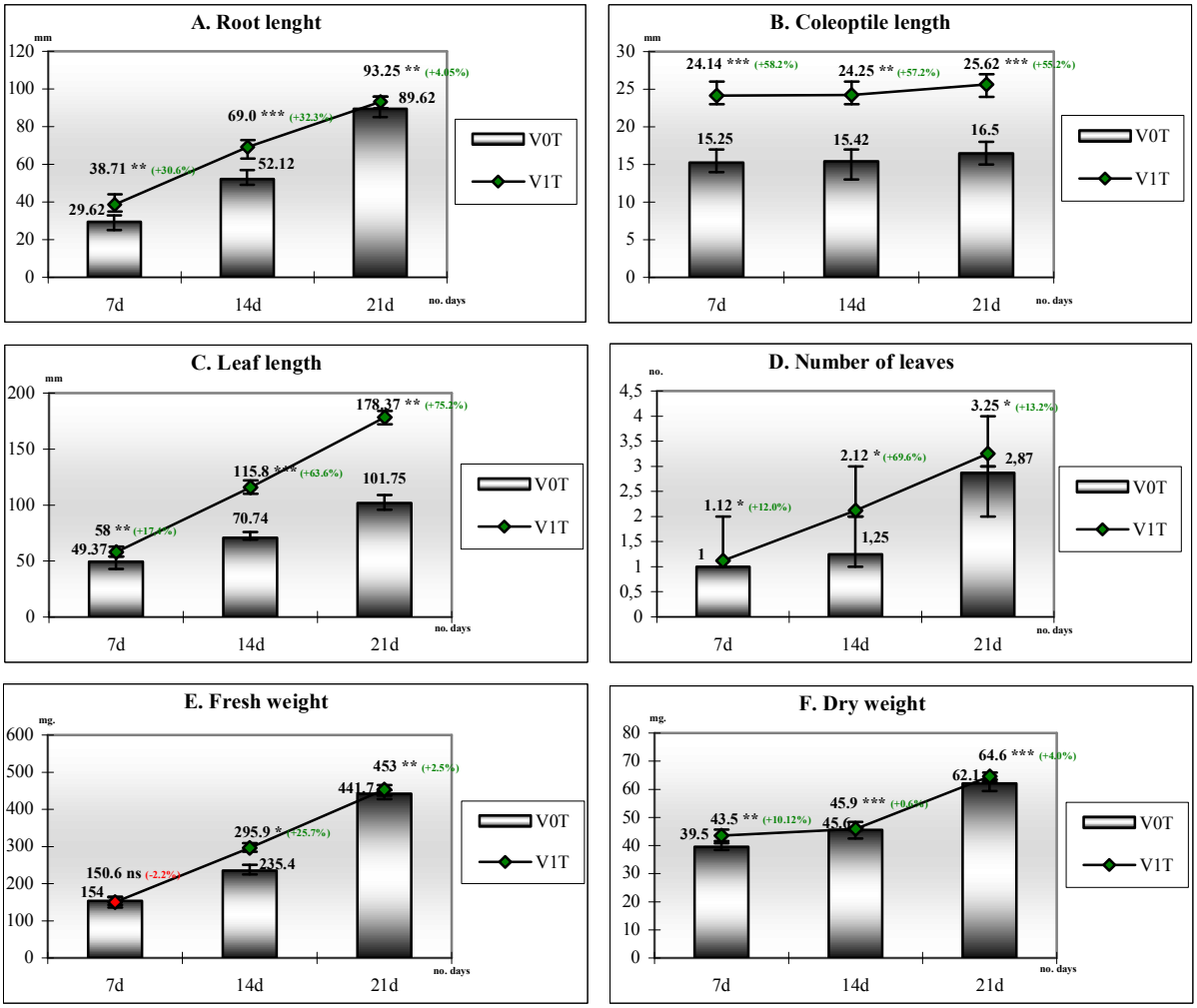

Figure 1. Comparative values of length of roots **(A)**, coleoptile **(B)** and length **(C)**, number of leaves **(D)**, fresh **(E)** and dry weight **(F)** / bottle at *Triticum aestivum* L.; the caryopses were placed either on MB-MS culture agarized media (var. **V_0T**) (control) either on liquid culture media provided in the latest case with filter-paper bridges, which also fulfilled the role of wick, being in contact with liquid culture medium (var. **V_1T**); $p>0.05$ = ns nonsignificant; $p<0.05$ * significant; $p<0.01$=** distinctly significant; $p<0.001$=*** very significant in comparison with control lot; the percentage represents the difference from the corresponding control.

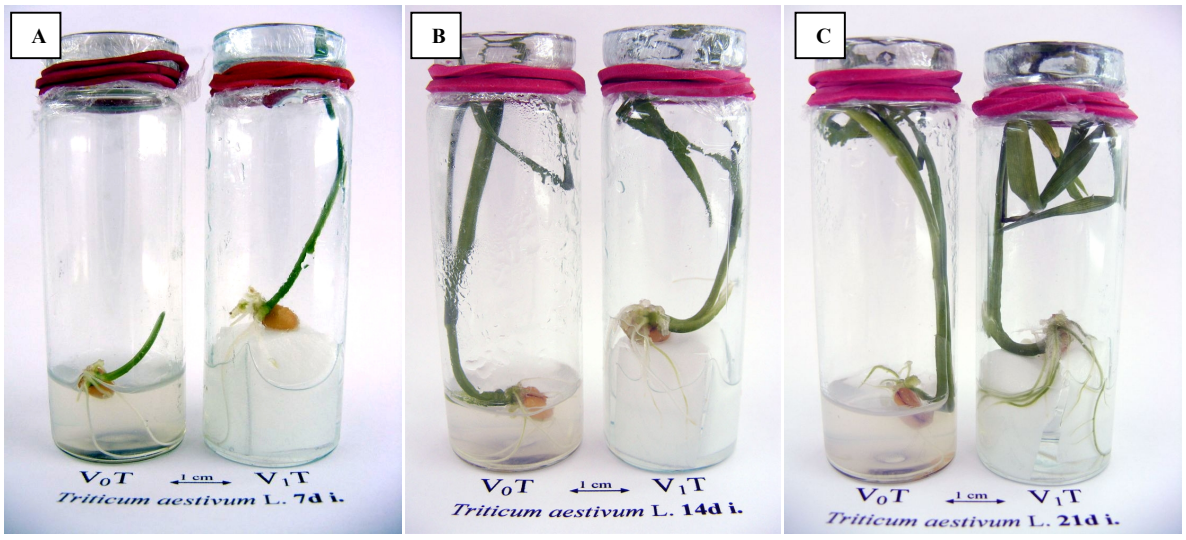

Figure 2. Comparision of *in vitro* macroscopic aspects at 7 days **(A)**, 14 days **(B)** and 21 days **(C)** in wheat; the caryopses was placed either MB-MS culture agarized media (var. **V_0T**) (control) or liquid media with same mineral and organic composition, in which case sustaining of inocules at the surface of culture media to avoid hypoxia is provided by a filter-paper bridge, which also fulfilled the role of wick, being in contact with liquid culture medium (var. **V_1T**).

difference was observed for the *number of leaf*, where for plantlets cultivated on filter-paper bridges developed 69.6% more leaves compared to control. High differences were also observed for the leaf *length*, considering 63.6% more compared to the control which in absolute values means a difference of 45.06 mm (a significant value). The smallest difference for these tested variants was obtained for *dry weight*, where for the new developed plantlets on filter-paper bridges, was only 0.6% higher compared to the corresponding control (Fig. 1A-F).

After *21 days*, it was possible to observe the first sign of senescence first at the level of leaflets and it is considered that this phenomenon is due to the lack of space in the jars, and this process is slightly increased for the experimental variant with filter-paper bridges (V_1T) because in this case the total length of the plantlets were significantly superior compared to control. Also, in both cases, the roots became partially green, but more pronounced in the group V_1T and the proliferative processes for callus production stopped, remaining at the dimension observed in the 14^{th} day for *in vitro* culture (Fig. 2C).

At this last stage of experiment observations, it was registered an acceleration of the growth in the *length of small leaves* for the V_1T variant (i.e. liquid medium with filter-paper bridges) compared to the control group V_0T (i.e. hydrogel type medium) with a difference of 75.2% (i.e. 76.62 mm higher, distinctly significant value) this value being the largest among the series of measured biometric and gravimetric parameters. Higher values compared to the control group were registered for the other analyzed parameters too, the smallest difference was recorded for the *fresh and dry weight*, with a differences of 2.5% (i.e. for distinctly significant value) respectively of 4.0% (i.e. significant value) (Fig. 1A-F).

DISCUSSIONS

Considering the results of this experiment, it is obviously that wheat plantlets grown on a liquid culture medium by using filter-paper bridges, showed a significant development advantage compared to the plantlets grown on a solid culture medium as a control medium. This may be explained if we consider that for the liquid culture medium – the use of filter-paper bridges increase the nutrients availability to the plantlets developed from the wheat caryospis. In the same manner, it can be added that through the *wick* characteristic of the filter-paper bridges, these inocula may take these nutrients in a selective manner. On contrary, in the case of solid culture medium, it can be considered that the selective easy access to the nutrients is restricted due to agar, the minerals and organic compounds are homogenously *trapped* in the agar meshes and during time it is establishes a gradient of concentration from the plantlets to the remote part of agarized medium. As a consequence, the nutrients become accessible only due to roots growth process which is not so fast and ready to be used such in the case of the liquid culture medium provided with filter-paper bridges.

This high efficiency of *in vitro* plantlets use when they are cultivated on liquid culture medium provided with filter-paper bridges compared to solid medium, was highlighted not only for wheat, where the total and embryogenic callus was doubled and significantly higher number of regenerants [29], but also to *Litchi chinensis* Sonne., probably because the rapid diffusion of phenolics in liquid medium prevented the accumulation of toxic levels [3]. Also, for orchids it was found that in a shake liquid culture medium the proliferation is generally faster and more extensive, but on filter-paper bridges the differentiation is always better [2]. For *Jasminum officinale* L., it was determined that the root emergence from the shoot base and later the root elongation it is facilitated by using liquid culture medium provided with filter-paper bridges in comparison with solid culture media [7]. The rooting process for fruit tissue culture systems using filter-paper bridges was also studied. For example, the rooting rates for *Zhumei crabapple* and strawberry grown on liquid culture media provided with filter-paper bridge were 20% and 4.1% more compared to controls (i.e. agarized culture media), and the rooting rate of cherry stock was the same as that of its control, but the rooting time was decreased to 3-10 days [51].

Providing a faster germination and growth of inocula on filter-paper bridges, respectively reducing the time by which vitroplants are reaching the optimum size for subcultivating or for acclimatization, results to a proportional saving of electricity. It is well known that the growth rooms conditions are very important and highly energy consumers because the technical parameters for *in vitro* culture imposes the maintaining of certain constant limits with an important consumption of electricity. Therefore, decreasing the time period in which are kept *in vitro* cultures under these new conditions, will decrease energy consumption, too. Or, today it is known that an important part of the amount of electricity used is produced by power plants, which are running on fossil fuels. Therefore, the use of filter-paper bridges may participate indirectly to the reduction of environment pollution, respectively is contributing for reducing the greenhouse gasses emissions.

Another advantage of using liquid culture medium provided with filter-paper bridges is the lower price compared to the solid culture medium. Agar as a solidifier agent used in the classical solid culture media has a higher price compared to the corresponding filter-paper, which may also be translated into an economic gain.

Aside all these advantages, a specific one is not to be neglected and it is related to acclimatization. It is well known that in the case of the agarized culture media after removing the roots from the nutrient substrate follows the carefully agar removal in order to avoid retention on the surface of agar. It is a complex process and recquire specialized hand care. Moreover, if some agar is still present on the root surface, during

acclimatization it can provide a perfect medium for bacteria or fungi installation which can impede the further development of plantlets.. In addition, the removal procedure of the roots from the agarized medium and the removal of this from the surface of the roots, can cause damages to this organ. In case of using a liquid culture medium provided with these filter-paper bridges, the paper removal is really easy and it avoids infection and other damages at the root system level of the acclimatized plantlets. Moreover, the process is very easy, clean and fast.

Based on these results it can be concluded that, the comprehensive protocol reported here is efficient, reproducible and with an important economic impact.

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