USING THE FILTER PAPER BRIDGE TECHNIQUE FOR THE INITIATION OF *IN VITRO* CULTURES OF MAIZE

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Abstract. Alongside wheat, maize is one of the most important species of cereals used for food and feed as well as in the bioethanol industry. As a result of this fact, maize is today the spotlight of many researchers, constantly trying to increase the productivity of this species, particularly important i from the economic point of view. The main aim of this article is to investigate the efficiency of the Blidar type filter-paper bridges (BFPB) in initiating the maize *in vitro* cultures for liquid culture media, in comparison with the conventional agarized culture media – solid culture media. In these experiments a modified Murashige-Skoog culture media (1962) (free of AIA and amino acids) supplemented or not with agar, were used. The inocula consisted in caryopsis of *Zea mays* L. (hybrid Kiskun 4255) and 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 5.34% for dry weight and 356.09% for leafs length.

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

INTRODUCTION

In bioethanol production at the global level, one of the most important crop plant species from economical point of view is represented by maize [24]. Also, maize is one of the most important crop globally because of its importance as food in the past and present [20]. According to FAO, at the global level, in 2000, were produced 592.47 million tons of corn on a total cultivated area of 137 million hectares and ten years later, the maize production increased at 844.4 million tons (with 42.5% more), obtained from a cultivated area of 161.9 million hectares (with 18.17% more) [15]. This demonstrates culture efficiency for the producers in economic term through a tendency to increase cultivated areas. This process may be explained through the demographic explosion we are coping today and the climate change policy, which means that there is an even more acute need of food on the hand and green fuel on the other hand [37]. Therefore, the prefered solution is to increase the cultivation efficiency of the existing surfaces, respectively using a sustainable bioeconomy [10, 30, 41], based on environment conservation and food biosecurity factors in relationship to climate change and ecosystem resilience [3, 4] that make up today's new positive paradigm of eco-bioeconomics [9], or inversely, in the worst case, using new surfaces arable land and continuing soil degradation [49].

Increasing the efficiency of productivity, assumes the creation of new plant varieties, new hybrids, resistant to more harsh environmental conditions [11, 36], which can be achieved in a relatively short time (months or years at most) using techniques provided by plant biotechnology and genetic engineering. In addition, the use of new techniques for decreasing *in vitro* cultivation time [13, 16], causes a parallel decrease in the energy consumption needed considering only the preservation of optimal physical conditions in the rooms of vegetation. Therefore, using cost-efficient *in vitro* culture techniques, both for conservation and for their multiplication for future use in various biotechnological or genetic engineering techniques, today, represents one of the major concerns for scientists [8, 39, 40].

As it is known, plant tissue and cells cultures, genetic engineering and genetic transformation techniques, essential contribute essential to enhance the germplasm quality in crop plants [42]. For each group of plants, the identification of a protocol of effective regeneration of the *in vitro* strains is essential for the efficient application of genetic transformation strategies [19]. Considerable work has been done on tissue culture and plant regeneration in maize primarily using immature tissues [38, 45, 51]. A series of researchers have focused on obtaining haploid plants of maize [12, 35], while other studies have been directed to the regeneration of callus from immature embryos [50] or from mature embryos [20, 21]. Also, at maize was followed the identification of innovative techniques on somatic embryogenesis, either from immature embryos [22, 28], either from callus [46]. Plant regeneration was obtained at maize, for example from immature embryos [1, 26, 27] from callus via somatic embryogenesis [17], or even from quality protein maize (QPM) [2]. In order to obtain more productive varieties of maize, there were used genetic transformation techniques, applied for example on immature embryos [43, 47], or directly on callus culture [14], and afterwards these scrientist developed the regeneration techniques after plant transformation [29]. All these studies were made *in vitro* culture conditions, which implied an initiation step of the *in vitro* cultures essential for an *in vitro* technique development.

Into the specialized literature, there are few references regarding *in vitro* culture initiation on liquid culture media provided with filter-paper bridges, but just few of them are referring to the advantages that this technique brings to initiate cultures in liquid compared to agarized medium. One of them, effectuated with wheat - as experimental model -

showed that plantlets grown on a liquid culture medium by using filter-paper bridges, a significant development advantage compared to the plantlets grown on a solid culture medium as a control medium [8]. In another study, regarding wheat, there was recorded too that both total and embryogenic callus were positively influenced in terms of growth which was doubled and significantly a higher number of regenerants were obtained on liquid culture media provided with filter-paper bridges compared to the cultivation on solid culture medium [32].

However, *in vitro* culture technique was used in a number of plant species, such as potato *Solanum tuberosum*, where it has been succeeded a conservation for three years of some micro-plants grown at low light and temperature conditions [31], at tabacco *Nicotiana tabacum* - in that case it has been achieved the regeneration of cell lines on this type of substrate [33], at alfalafa *Medicago sativa* - where the initiation of the *in vitro* cultures was performed on filter paper bridges, in order to determine if genetic differences in winter hardiness between dormant and non-dormant alfalfa and to determine the physiological and biochemical bases for differences in freezing tolerance of suspension cells [23]. 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 poplar *Populus euphratica* [48], at chickpea *Cicer arietinum* too, to study the role of enzymes and identification of stage-specific proteins in developing somatic embryos [25], but also at two genotypes of ornamental strawberry *Fragaria* x *Potentilla* to studying the regenerative capacity of the callus [44].

This study confirm and support our previous research results already published in our laboratory [8], where it describes in detail the cost-efficient method to initiate *in vitro* cultures, using filter-paper bridges for liquid culture media.

MATERIALS AND METHODS

Plant material and growth condition. Uniform and healty mature caryopsis of maize (*Zea mays* L. subs. *mays*) hibrid "Kiskun 4255" were selected for this experiment and kept under running tap water for 15 min. Plant material was disinfected using a 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. In the next step, the caryopsis were cultivated aseptically on a Murashige – Skoog (1962) [34] (MS) modified culture medium (e.g. aminoacids and hormone free), either agarized (with 7 g/l agar-agar) – V_0Z experimental variant (control), either liquid, inocula was supported at the surface using a Blidar type filter paper-bridge (BFPB) [7] (V_1T variant). The medium pH was adjusted to 5.7. After inoculation, 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 for 21 days.

Growth measurement. As in our previous study [8], 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 on the control group (V_0Z) was considered as 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 Statistical Package for Social Sciences (SPSS, version 16.0).

RESULTS

At *7 days* from the beginning of the experiments, in the case of caryopsis placed on BFPB, the newly formed vitro plants showed an higher growth compared to those on agarized culture media, both at the basement part of the small roots and shoot, too. It is obviously to notice that for the generated vitroplantulele on BFPB, adventitious roots appeared (Fig. 2A).

At this time of the experiment, the mean values of all the studied parameters were superior for medium variant on BFPB (V_1Z) compared with those obtained on the agarized culture medium (V_0Z). The highest difference was observed for the *length of the root* (47.11 mm, with 179.7% superior compared to the control – a very significant value), and the lowest for the amount of *fresh weight* of the plant's biomass (930.3 mg, 12.1% higher than the witness) (Fig. 1A-F; 2A). After only 7 days from inoculation, significant differences in growth of the *in vitro* plantlets on the two types of nutrient substrate were observed.

At *14 days* of *in vitro* culture, regarding the morphological aspect, it the case of 45% of *in vitro* plantlets regenerated on the agarized substrate, an early calusogenesis phenomenon occurred. The callus was senescent, but of friable regenerative type. It was extended starting with the base of the roots, for a short length of 3-4 mm; callus thickness being of at most 1 mm. In case of the *vitro* plantlets on liquid culture medium with BFPB, the genesis of callus was recorded in 12.1% of regenerant. In this case, the layer of callus was extended on a length of 4-5 mm from the roots, about 80% being of embriogenic type. As a special phenomenon for 9.7% of *in vitro* plantlets of V_1Z variant, the small roots became sligthly pigmented in red and iti is suspected that it might be due to anthocyanin's accumulation (Fig. 2B).

The above presented phenomena and referred at 7 days were emphasized in terms of biometric and

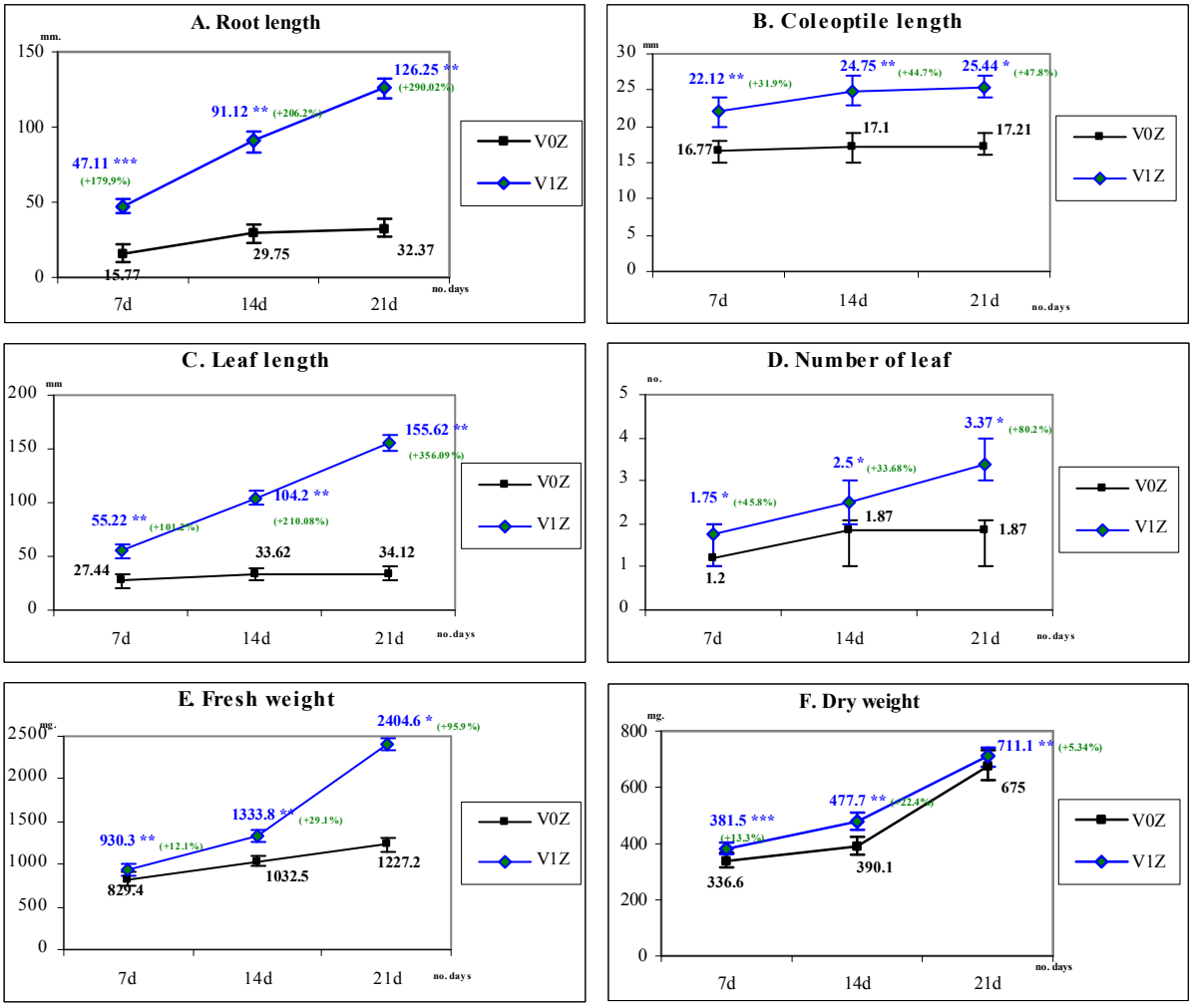

Figure 1. Comparative values of length of roots **(A)**, coleoptile **(B)** and length **(C)**, number of leaves **(D)**, fresh **(E)** and dry weight **(F)** / bottle at *Zea mays* L. subs. *mays*; the caryopses were placed either on MB-MS culture agarized media (var. **V_0Z**) (control) either on liquid culture media provided in the latest case with BFPB, which also fulfilled the role of wick, being in contact with liquid culture medium (var. **V_1Z**); 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

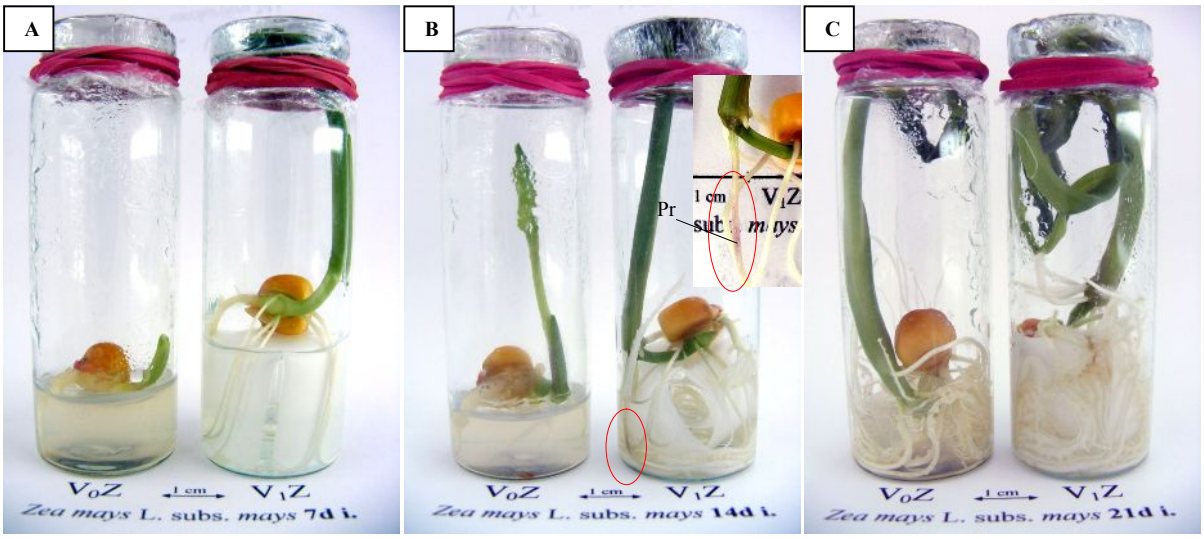

Figure 2. Comparision of *in vitro* macroscopic aspects at 7 days **(A)**, 14 days **(B)** and 21 days **(C)** in maize; the caryopses was placed either MB-MS culture agarized media (var. **V_0Z**) (control) or liquid media with same mineral and organic composition, in which case sustaining of inocules at the surface of culture media to avoid hypoxia is provided by BFPB, which also fulfilled the role of wick, being in contact with liquid culture medium (var. **V_1Z**); Pr – pigmented root

gravimetric parameters values, respectively the differences corresponding to control, were amplified. The highest difference was observed for the *leaf length* parameter, where for plantlets cultivated on BFPB were with 210.08% higher compared to control (distinctly significant value). High differences were also observed for the *root length*, considering 206.2% more compared to the control which in absolute values means a difference of 91.1 mm (distinctly significant value). The smallest difference for these tested variants was obtained for *dry weight* of the biomass, where for the new developed plantlets on BFPB, was with 22.4% higher compared with the corresponding control (Fig. 1A-F).

Although the volume within in the culture vessels was insufficient, the *in vitro* plantlets kept their viability even at the *21st days*, both on agarized substrate (V_0Z) and the experimental variant with BFPB being in contact with liquid culture media (V_1Z) (Fig. 2C). However, only 29% of regenerants showed a higher growth on V_0Z variant, compared to 80.6% of those growth on V_1Z variant, the *in vitro* plantlets with multiple roots, highly branched, white or, here and there, to the *in vitro* plantlets initiated on BFPB, their roots showed a slight red pigmentation (12.9%), as described above; about the genesis of callus, we can specify that it was much firms in the sample group (V_1Z) compared to the control (V_0Z), but in both cases the newly formed leaflets were green. The callusogenesis process was present in 45% of vitro cultures of control variant and only at 12.9% in case of liquid medium with BFPB variant, located either at root level in case of V_0Z, or to the embryo level and the root at V_1Z; in both cases, the callus was yellowish, presenting signs of early senescence.

At this last stage of the experiment's observations, it was registered an acceleration of the growth in the *length of small leaves* for the V_1Z variant (i.e. liquid medium with BFPB) compared to the control group V_0Z (i.e. hydrogel type medium) with a difference of 356.09% (i.e. 155.6 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 *dry weight*, with a differences of 5.3% (i.e. for distinctly significant value) (Fig. 1A-F).

We can therefore say therefore, the use of BFPB proved to have beneficial effects on the growth of maize *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 supported further in this article.

DISCUSSIONS

Considering the results of this experiment, it is obviously that the neoformed maize plantlets grown on a liquid culture medium by using BFPB, showed a significant development advantage compared to the plantlets grown on a solid culture medium as a control medium. As well as in the previous study (see Blidar *et al.*, 2011 [8]), this may be explained if we consider that for the liquid culture medium – the use of BFPB 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 BFPB, these inocula may take these nutrients in a selective manner. On contrary, in the case of the solid culture medium, it can be considered that the selective easy access to the nutrients is restricted due to the agar, the minerals and organic compounds that are homogenously *trapped* in the agar meshes. As a consequence, nutrients are accessible only as the environment is entirely consumed, respectively all minerals and organic components, which makes no selective absorption.

This higher efficiency of the *in vitro* plantlets recorded when they are cultivated on liquid culture medium provided with BFPB compared to the solid medium, was highlighted not only for wheat, where the total and embryogenic callus was doubled and significantly higher number of regenerants [32], but also, in terms of root, coleptile and leaflets length, leaf number and fresh and dry weight, the values were higher than in the case of the agarized culture media [8], but also in other plants, too. Thus, for example, at *Jasminum officinale* L., it was determined that the root emergence from the shoot base and later the root elongation are facilitated by using liquid culture medium provided with BFPB in comparison with the solid culture media [6]. At *Zhumei crabapple* it was demonstrated that the *in vitro* plantlets which grow on liquid culture media provided with BFPB were 20% and 4.1% more compared to the 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 [52]. In case of the *Litchi chinensis* Sonne species, the explanation for the higher efficiency of the BFPB substrate, was considered to be given probably because of the rapid diffusion of phenolics in liquid medium preventing the accumulation of toxic levels [5].

Reducing the time by which vitroplants are reaching the optimum size for subcultivation or for acclimatization by using the Blidar type filter paper-bridge technique [7] (BFPB), providing a faster germination of the seeds and also a faster growth of the roots and shoots of generated plants *in vitro*, results to a proportional saving of energy. The growth rooms' conditions are very important for vitroplants. They are kept under constant limits with an important consumption of energy (i.e. electricity, methan gas, etc.). Therefore, decreasing the time period in which are kept *in vitro* cultures under these new conditions, will decrease energy consumption, too. Therefore, the use of BFPB may participate indirectly to the reduction of environmental pollution, respectively is contributing in the reduction of the greenhouse gasses emissions [8].

Another advantage for using liquid culture medium provided with BFPB is the lower price compared to the solid culture medium [8]. 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 the acclimatization, which can be done without the risk of damaging the rootlets during procedures of agar removal from their surface, prior to the acclimatization. Also, it is avoided the possible infections at the rootlets level, because of the not properly removed agar which can provide a perfect medium for bacteria or fungi installation, since it is not present in the liquid culture medium provided with filter paper bridges.

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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