Successful Regeneration of Sweet Pepper (*Capsicum Annuum* l.) in an Airlift Bioreactor: Effect of Medium Phase and Genotype

S. Grozeva[1], V. Rodeva[1] and S. Kintzios[2*]

[1]*Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria*

[2]*Faculty of Agricultural Biotechnology, Agricultural University of Athens, Athens, Greece*

Abstract: We investigated the regeneration of two Bulgarian sweet pepper cultivars ('Hebar' and 'Stryama') on solid and liquid culture medium. Liquid cultures were incubated in temporary immersion RITA® airlift bioreactors. During a period of 60 days, culture in the liquid medium was associated with significantly increased shoot tip growth, number of lateral shoots, percentage of rooted plants and micropropagation coefficient, with essentially no callus formation. Regenerants from cultivar 'Hebar' exceeded those of cultivar 'Stryama' in all measured traits. A coefficient of micropropagation equal to 5.3 was observed for the superior cultivar. The results of the present study manifest the possible scale up of the micropropagations of this recalcitrant species, resulting in higher number of vigorous plants within a short period of time.

Keywords: Airlift bioreactor, ferulic acid, micropropagation, organogenesis, sweet pepper, Vitamin B_{12}.

INTRODUCTION

Pepper is an important vegetable crop grown worldwide. Unlike other Solanaceous species, *Capsicum annuum* L. is considered as a recalcitrant species with regard to its capacity for *in vitro* regeneration. Also, the inefficient development of induced buds into complete plantlets prohibits the use of micropropagation in pepper genetic improvement programs [1, 2]. Successful pepper micropropagation is restricted in a few cases only [3-5]. An effective system for mass-scale micropropagation would greatly assist sweet pepper breeders in the preservation and maintaining of elite plants, sterile and transgenic plants and F_1 hybrid plants displaying heterosis [6, 7].

Bulgaria is well known for its diversity of pepper genotypes, while adapted local forms are distinct from popular cultivars in Western Europe, therefore constituting a promising breeding material [8]. In the present study, we investigated the regeneration of two Bulgarian pepper cultivars, based on plant regeneration from shoot-tips explants. Furthermore, we compared the regeneration response on to solid medium and in liquid medium (in an airlift bioreactor), in an attempt to scale-up the regeneration of this important horticultural species.

MATERIALS AND METHODS

The Bulgarian pepper cultivars Stryama and Hebar were used in the present study. Stryama is higher than Hebar (plant height 54-60 cm with 3-4 branches, compared to 45-50 cm with 2-3 branches, respectively). Stryama is cultivated for early and mid-early production, with fruits suitable for fresh consumption and canning. The pericarp is tender (5-6 mm long), sweet with thin skin and pleasant fragrance. The average yield is 40-45 t/ha. Hebar is cultivated for super early to mid-early production. The pericarp is tender (6 mm long) and the average yield is 30-45 t/ha.

Seeds of both cultivars were surface sterilized in 5% (w/v) calcium hypochlorite solution for 20 minutes and then were rinsed three times with sterile distilled water and cultivated aseptically *in vitro* on half-strength MS medium [9] supplemented with 2% (w/v) sucrose and 0.7% (w/v) agar for producing of shoot tip explants. Shoot tips (12-15 mm long) were excised from the seedlings and incubated on above mentioned MS medium supplemented with 3% (w/v) sucrose, 0.1 mg L^{-1} IAA, 0.1 mg L^{-1} vitamin B_{12}, 5.0 mg L^{-1} ascorbic acid and 5.0 ml l^{-1} huminic acid and solidified with 0.7% (w/v) agar (MS1). This medium has been previously [8] identified as optimal for plant regeneration from shoot tips of the investigated pepper varieties. Media were adjusted to pH 5.8 using 1N NaOH or 1N HCl, autoclaved at 121°C for 20 min and poured into 250 ml glass vessels. For liquid culture, explants were incubated in MS1 medium without agar in temporary immersion RITA® air lift bioreactors (Vitropic, France) with an internal culture volume of 1l. Air was supplied to the culture chamber at a rate of 1l m^{-1}, at 0.2 bar. Immersion took place for 15 min with 10 min intervals. Both solid and liquid cultures were incubated at 25 ± 1ºC, a photosynthetic proton flux density (PPFD) of 200 µmol m^{-2} s^{-1} and 16/8 h photoperiod. Shoot tips (3 cm long), having the first internode with leaves were excised from regenerated plants and cultivated for subsequent regeneration in either solid or liquid medium. In total, three subcultures were done, each lasting 20 days. Prior to each subculture, individual regenerants were separated from a cluster containing 2-6 plants.

*Address correspondence to this author at the Faculty of Agricultural Biotechnology, Agricultural University of Athens, Athens, Greece Tel. +302105294292; Fax. +30 210 5294286; E-mail: skin@aua.gr

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Table 1. Results of a Two--Way Analysis of Variance for Micropropagation Depending on Genotype (Factor A) and Medium Phase (Factor B)

Source of Variation	Relative Effect Size (% of Total Variance)
Genotype (A)	10.97***
Medium phase (B)	85.78***
A x B	1.53*
Error	1.72

Level of significance: * = $p < 0.05$, *** = $p < 0.001$.

Each experiment was carried out twice in three replications, each with 20 explants. The length of the microplants, percentage of rooted plants and the number of lateral shoots were recorded at the end of the 60-days culture period (including all subcultures). For each subculture, the coefficient of micropropagation was calculated as the number of regenerated shoots from an initial explant X the percentage of rooted plants. Subsequently, the average coefficient of micropropagation for the total experimental period (three subcultures) was calculated. Data were analyzed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Both the phase (solid/liquid) of the culture medium and the plant genotype affected the regeneration of sweet pepper plantlets via adventitious shoot organogenesis (Table **1**). For both cultivars used in the present study, culture in the liquid medium was associated with significantly increased shoot tip growth (Fig. **1**), number of lateral shoots (Fig. **2**), percentage of rooted plants (Fig. **3**) and average micropropagation coefficient (Fig. **4**). Regarding genotypical effects, regenerants from cultivar 'Hebar' exceeded those of cultivar 'Stryama' in all measured traits.

Essentially no callus formation was observed in the base of stem explants used for culture initiation. This is noteworthy in view of the fact that unorganized callus from pepper explants is not capable of shoot bud induction and subsequent regeneration [10,11], while teratological proturberances frequently appear during plant regeneration from sweet pepper explants cultured on medium supplemented with IAA [5]. It is possible that addition of ferulic acid to the culture medium promoted the formation of organized structures [8], although genotypical effects might have contributed, as well [12, 13]. Therefore, the results of the present study manifest the possible use of the adventitious shoot induction for true-to-type clonal propagation of sweet pepper cultivars. In addition, and during each subculture, root induction was observed simultaneously to shoot induction (Figs. **5-8**), so that the necessity for a rooting stage (e.g. by subculturing on rooting medium) was omitted.

Mass regeneration of Bulgarian pepper cultivars was optimized in the conditions of temporary immersion system of air-lift bioreactors which increased the average micropropagation coefficient in the both cultivars Stryama and Hebar (3,1 to 5,3) compared with the coefficient observed in solid cultures. Moreover, bioreactor-derived microplants were very vigorous and developed broad leaves, stronger roots and stems (analytical data not shown). However, we observed a decrease of the coefficient of micropropagation with increasing number of subcultures. For example, the coefficient at the end of the third (and final) subculture for regeneration in liquid medium was 3.2 for Stryama and 4.1 for Herbar, i.e. much lower than the average coefficient.

Increasing of the effectiveness in the regeneration process by developing of higher multiplying coefficients is

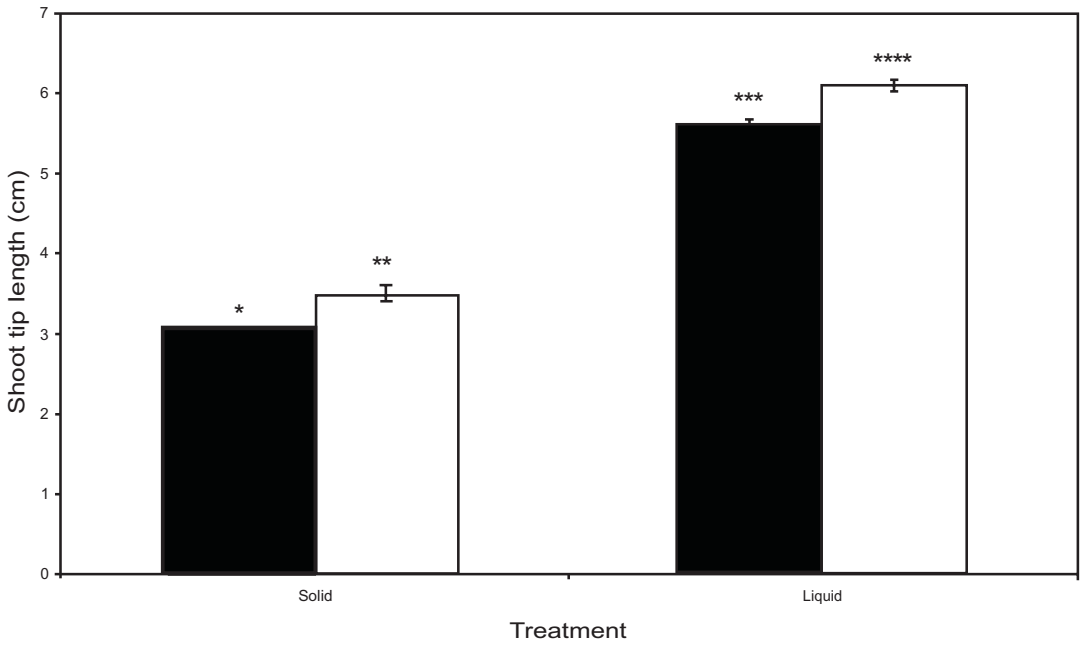


Fig. (1). Effect of the state (solid/liquid) of the culture medium on the length of shoot tip explants from the sweet pepper cvs. Stryama (closed columns) and Hebar (open columns) (n = 60 replications and error bars represent standard errors of the average value of all replications, values followed by different number of asterisks are statistically different at $p<0,05$).

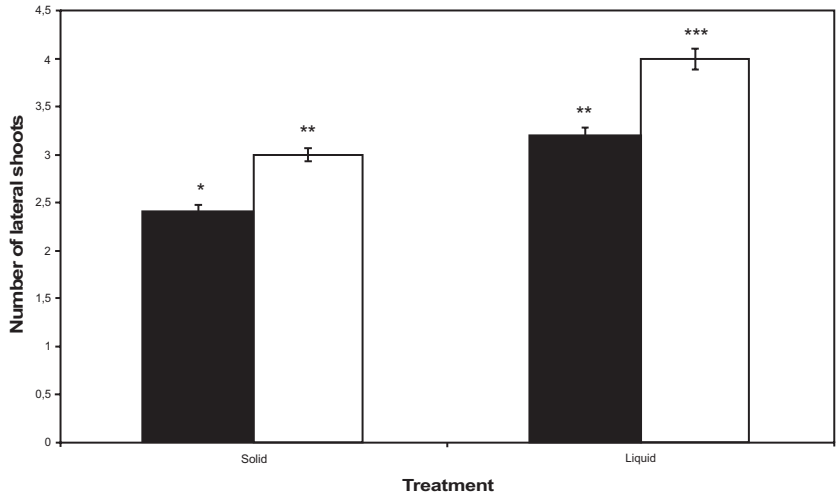


Fig. (2). Effect of the state (solid/liquid) of the culture medium on the number of later shoots/shoot explant regenerated from the sweet pepper cvs. Stryama (closed columns) and Hebar (open columns) (n = 60 replications and error bars represent standard errors of the average value of all replications, values followed by different number of asterisks are statistically different at p<0,05).

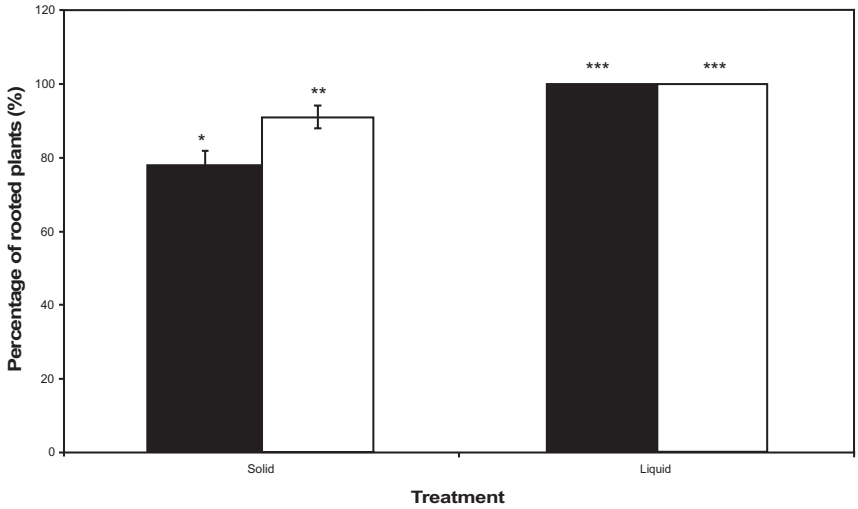


Fig. (3). Effect of the state (solid/liquid) of the culture medium on the percentage of rooted regenerants from the sweet pepper cvs. Stryama (closed columns) and Hebar (open columns) (n = 60 replications and error bars represent standard errors of the average value of all replications, values followed by different number of asterisks are statistically different at p<0,01).

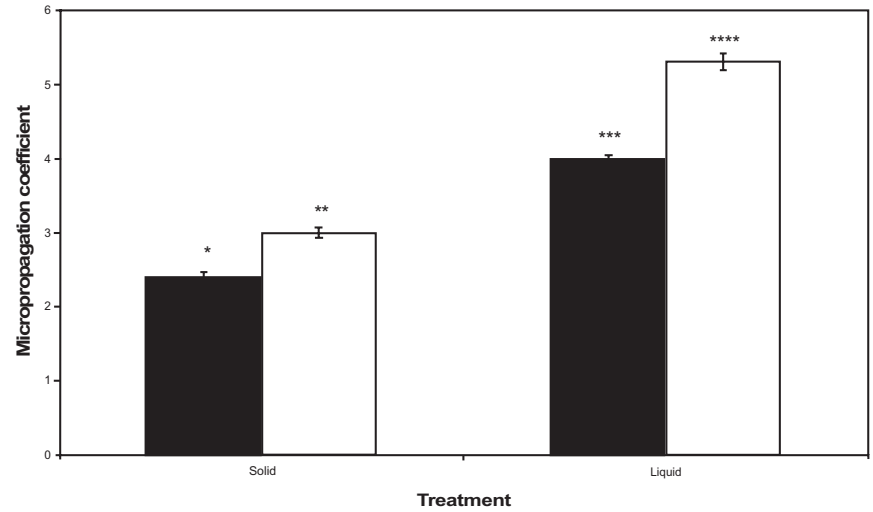
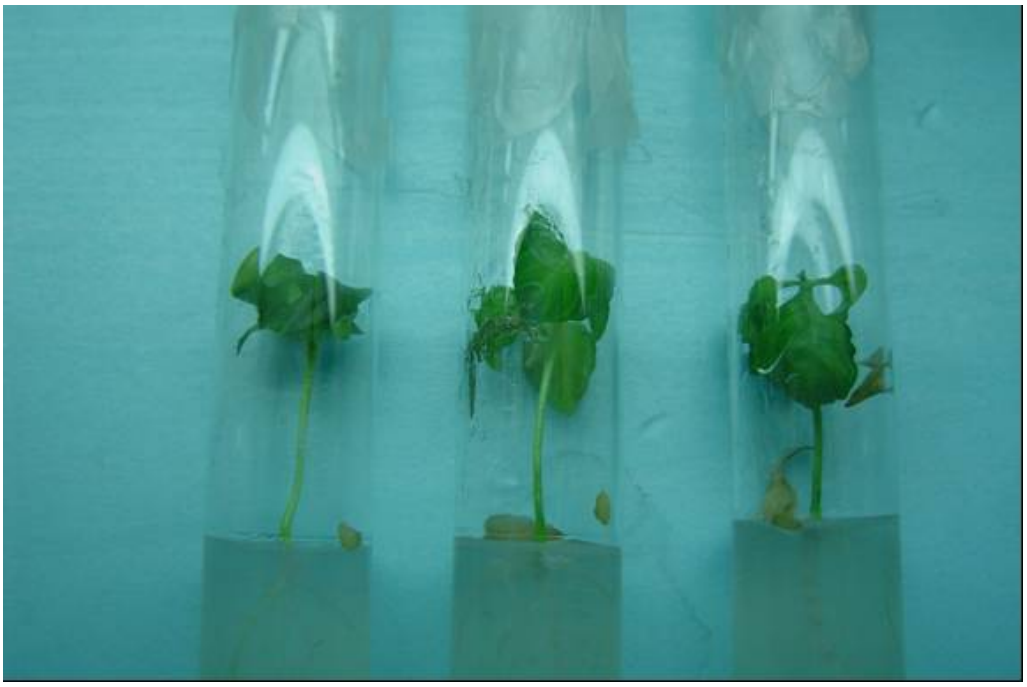


Fig. (4). Effect of the state (solid/liquid) of the culture medium on the average coefficient of micropropagation of the sweet pepper cvs. Stryama (closed columns) and Hebar (open columns) (n = 60 replications and error bars represent standard errors of the average value of all replications, values followed by different number of asterisks are statistically different at p<0,05).



F**ig. (5).** Regenerated pepper plants (cv. Hebar) subcultured on solid MS1 medium, after separation from a cluster containing 2-6 plants.

Fig. (6). Regenerated pepper plants (left: cv. Hebar, right: cv. Stryama) subcultured on solid MS1 medium, after separation from a cluster containing 2-6 plants.

Figs. (7, 8). Comparison of regenerated pepper plants (cv. Stryama) on liquid (left, in bioreactor) and solid (right) MS1 medium, after their separation from a cluster containing 2-6 plants.

achieved in many plant species by using of liquid culture micropropagation systems in bioreactors [14]. In liquid medium, the close contact of the tissue with the medium may stimulate and facilitate the uptake of nutrients and phytohormones leading to better shoot and root growth [15]. An acceleration of *in vitro* plant growth in liquid medium, in particular in an aerated reactor vessel has been previously reported for a number of species, including cucumber [16] and sweet basil [17]. In these cases, increased plant regeneration has been associated with a rapid turnover of primary metabolites.

In conclusion, the present study is a first step towards establishing a successful regeneration protocol of Bulgarian sweet pepper in an airlift bioreactor in order to scale up the regeneration process, resulting in higher number of plants within a short period of time. Further research will be focused on acclimatization of the regenerants, as well as investigation of the response, to the same culture conditions, of widely established commercial cultivars.

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ABBREVIATIONS

IAA	=	3-indoleacetic acid
MS	=	Murashige and Skoog basal medium
PGR	=	Plant growth regulator
PPFD	=	Photosynthetic photon flux density

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