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NEW PROTOCOL FOR *IN VITRO* PROPAGATION OF BERRY PLANTS BY TIS BIOREACTOR

L. GEORGIEVA[1*], I. TSVETKOV[1], M. GEORGIEVA[2] and V. KONDAKOVA[1]
[1] *Agricultural Academy, Agrobioinstitute, BG-1164 Sofia, Bulgaria*
[2] *Agricultural Academy, Institute of Mountain Agriculture and livestock, BG-5600 Troyan, Bulgaria*

Abstract

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The purpose of this study is to explore the possibility of optimizing the standard protocols for in vitro propagation of important berry crops: raspberry, cv. Polka and strawberry, cv. Tudla, using bioreactor system. The effectiveness of the two systems (solid media and liquid in TIS bioreactor, RITA® type) are evaluated in 2 stages – propagation (on MS based media supplement with 0.5 mg.l^{-1} BAP, 0.05 mg.l^{-1} IBA, 0.03 mg.l^{-1} GA_3 and 30 g.l^{-1} sucrose) and rooting (MS without hormones supplement with 30 g.l^{-1} sucrose). At each stage are measured fresh and dry weights of the explants, while the rest continued their development. In solid medium strawberry plants multiplication ratio for 4 weeks was 1.9 while in liquid – 4.1. Respectively for raspberry this ratio was 3.2 and 6.7. Fresh mass of TIS plants were increased for both species, whereas in plants grown on solid medium was observed a much higher percentage of dry matter. This means that the high fresh weight of the plants in a liquid medium is primarily due to water accumulation. Root initiation of strawberry began about 3rd week and after 5th week all plants was rooted successfully in both liquid and in the solid medium. For the same time only few raspberry plants formed roots in the same condition. That is the reason we tested another 3 variants of rooting systems. All plants of the three variants are rooted successful within 4 weeks. Highest percent dry weight, where the plants transferred on solid medium from liquid one (variant 2). Rooted plants were successfully adapted in soil in a greenhouse. TIS is a promising alternative system of mass propagation for strawberries and raspberries. The different purposes of application of the bioreactor system requires further optimization of the growing medium and precise conditions of cultivation of each species and variety. Micropropagation for strawberry could be entirely in liquid medium. For raspberry there is a problem with hyperhydration when the plants stay more time in liquid medium. We suggest an optimized protocol for mass propagation of raspberries, which includes plant propagation in a liquid medium (TIS bioreactor) and rooting them in solid medium.

Key words: TIS bioreactor, *in vitro* propagation, strawberry, raspberry
Abbreviations: TIS – Temporary Immersion System; TDZ – Thidiazuron; MS – Murashige & Skoog, 1962; BAP – 6-Benzylaminopurine; IBA – indole butyric acid; GA_3 – Gibberellic acid; PTFE – Polytetrafluoroethylene

Introduction

In vitro propagation has been proved as a highly efficient method for production of large quantities of true to type and free of diseases plants with minimum use of space and less initial plant material. However, the application of traditional micropropagation, using solid medium is today still limited in commercial production mainly due to its intensive labor input and thereby expensive (Welander et al., 2014).

As opposed to the traditional micropropagation, semi-automatic bioreactors use liquid media based on the temporary immersion principle. Temporary Immersion System (TIS) was created in the 80's (Harris, 1983). The principle of TIS technology is that plant material is immersed in liquid growth

**Corresponding autor*: lilk81@abv.bg

media for short periods at regular intervals. These immersions are sufficient for the plants to take up the nutrients. TIS technology makes use of the advantages of liquid cultures, while growing the plant material under high gas-exchange environments. The semi-automatic bioreactor system has been considered as a promising alternative to the solid culture system at lower price (Mbiyu at al., 2012; Welander et al., 2014).

One of the most popular model TIS bioreactor is the RITA system (Vitropic, France), firstly introduced by Alvard et al. (1993). Since then it has been used in several scientific studies (Zhu et al., 2005; Pavlov and Bley, 2006; AL-Mayahi, 2015). This system is composed of a container with two compartments that are placed on top of each other (Etienne and Berthouly, 2002). The plant material is located on the top compartment and the medium is in the bottom compartment. Medium immersion over explants is then performed through application of an air pressure to the lower compartment, so, that the medium level rises and reaches the plant culture level. The air pressure is also functioning as ventilation due to bubbles that are created during this process. Excessive air can escape from the container through a ventilation tube connected with a filter (Welander et al., 2014).

Strawberry (*Fragaria × ananassa*) and raspberry (*Rubus idaeus*) are economically important berry crops for Bulgaria.

There are few reports on *in vitro* bioreactor culture of strawberry (Takayama and Akita, 1998). Keßler et al. (1997) cultured strawberry cell suspensions in liquid MS medium. Hanhineva et al. (2005) obtained shoot regeneration from leaf explants of five strawberry cultivars in commercially available TIS bioreactors (RITA®). They reported that regeneration and development of plantlets was better on semi-solid media, but the time taken for handling plant material for cultivation in the TIS system was less than half that required on semi-solid medium. Debnath (2008a) developed a protocol for adventitious shoot regeneration, proliferation and rooting of strawberry (cv. Bounty), using a TIS bioreactor (RITA®) system in a liquid medium combined with *in vitro* culture on semi-solid gelled medium. In liquid medium, TDZ supports rapid shoot proliferation at low concentration (0.1 μM), but induces hyperhydricity (Debnath 2008a).

The raspberry micropropagation efficiency in liquid media and on semi-solid media is genotype dependent (Zawadzka and Orlikowska, 2006; Debnath, 2010; Debnath, 2010), because of different endogenous levels of plant growth regulators (PGRs) and additional variations in receptor affinity or cellular sensitivity to PGRs (Minocha, 1987). Rooting of *in vitro* shoots as cuttings is not successful (Debnath, 2010). Bioreactor-multiplied hyperhydric shoots were transferred to gelled medium and produced normal shoots within 4 weeks of culture (Mulwa and Bhalla, 2000; Debnath, 2011).

The purpose of this study is to explore the possibility of optimizing the standard protocols for micropropagation of important berry crops: raspberry, cv. Polka and strawberry, cv. Tudla, using bioreactors.

Materials and Methods

Plant material and standard cultivation

For our experiments, we used well-developed plantlets from strawberry and raspberry, growing under *in vitro* conditions. ABI's standard protocol for *in vitro* micropropagation of raspberries and strawberries including two stages: multiplication on "Pr" medium and growth and rooting on "R1" medium (Table 1).

Experimental design

The initial *in vitro* plants are separated into uniformed individual shoots by removal of all fully developed leaves (Figure 1a, b)

The first stage of the comparison between the two systems (solid medium in glass jars and a liquid medium in a TIS bioreactor) included placing of 30 plants from the two cultures on medium for the propagation (Pr) in both types of conditions. The medium formula for liquid and solid propa-

Table 1
Components of nutrient media*

Media	Components			
	Pr (solid/liqid)	R1 (solid)	R2(solid/liqid)	R3 (liqiud)
Basic medum MS+vit	full strength	full strength	full strength	½
BAP, mg l^{-1}	0.5	–	–	–
IBA, mg l^{-1}	0.05	–	–	0,2
$GA_{3,}$ mg l^{-1}	0.03	–	–	–
Sucrose g l^{-1}	30	30	30	20
Activated charcoal	–	2	–	–
agar g l^{-1}	8 /–	8	8/–	–

*The pH was adjusted to 5.8 before autoclaving. Autoclaving of media was performed at 121ºC and 1.2 bars for 20 minutes.

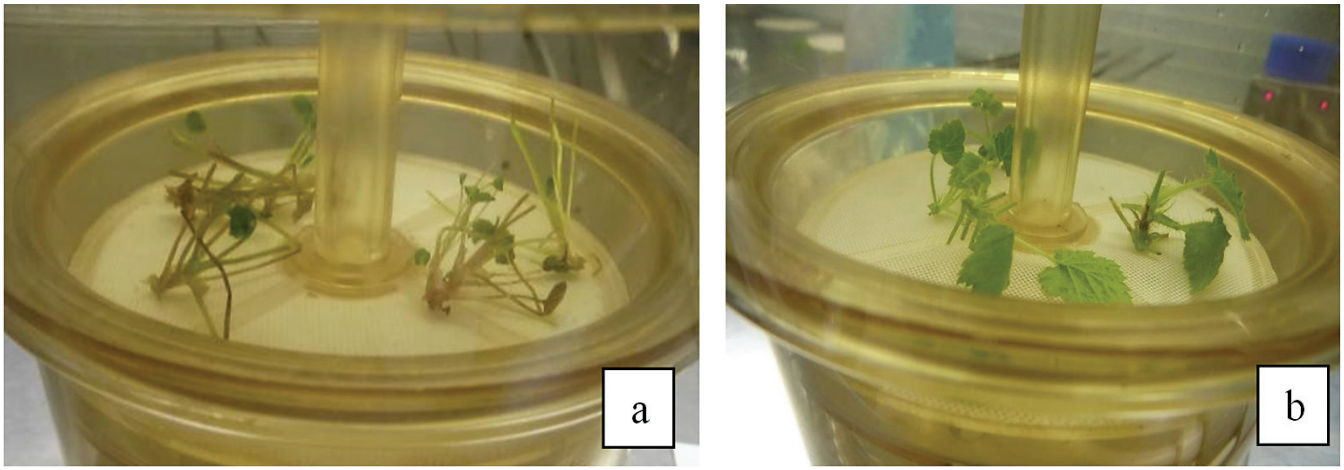

Fig. 1. Initial explants at the beginning of the experiment, placed in RITA bioreactor: *a* – strawberry, *b* – raspberry

gation was the same as described above (Tabl.1), except for addition of 8 g l^{-1} agar to the solid media in the glass jars.

The bioreactors were commercially available TIS bioreactors – RITA®, (described by Georgiev et al., 2014) were autoclaved connected with sterile PTFE filters, filled with 200 ml of the appropriate medium and the explants in a sterile environment. Liquid medium immersion was performed once per 24 hours for 20 min. All bioreactors contained 7 individual shoots each. Comparing *in vitro* cultures on solid medium included the same initial amount of shoots and was treated under the same conditions as the bioreactors.

The *in vitro* cultures were maintained at 21°C with 16/8 h photoperiod (2500 lux) of white fluorescent light.

Four weeks later, the number of new shoots for each explant was recorded and some of the plants were used for measuring fresh and dry weight. The rest were divided into individual stems and are transferred to the rooting medium R2, liquid and solid, respectively (Tablle 1) for four weeks.

The shoots production was evaluated at the end of multiplication stage after four weeks cultivation.

Fresh and dry weights were measured at the end of each stage. The dry weight was examined after being dried in forced air at 40ºC for 48 hours.

Evaluation was carried out for both bioreactors and solid medium cultures.

Because of the poor rooting of raspberries on first rooting stage, both – liquid and solid media R2, we tested different variants of rooting systems.

Variant 1. Plants from solid R2 medium were transferred to solid R1 medium

Variant 2. Plants from liquid R2 medium (from bioreactor) were transferred to solid R1 medium.

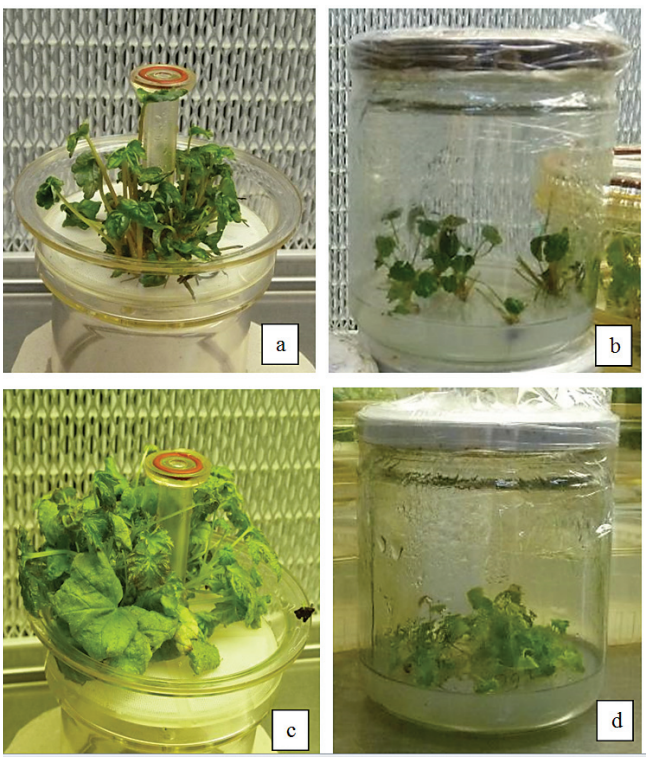

Fig. 2. Plants after 4 weeks cultivation on Pr medium (Tab. 1)
***a* – strawberry in RITA,**
***b* – strawberry in solid medium,**
***c* – raspberry in RITA,**
***d* – raspberry in RITA**

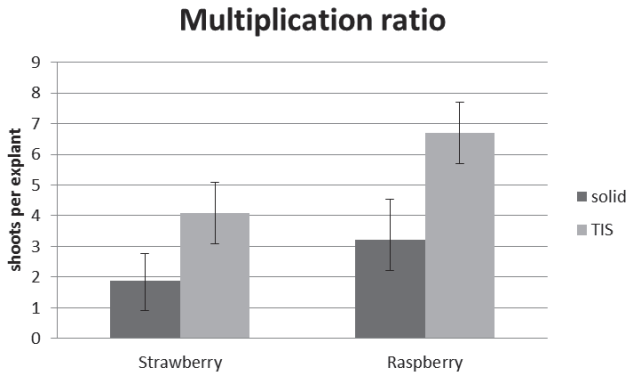

Fig. 3. Shoot multiplication (average mean) for strawberry (cv. Tudla) and raspberry (cv. Polka) in solid media and TIS bioreactor (Error bars indicate the standard deviation of total number of shoots)

Variant 3. Plants from solid R2 medium were transferred to a bioreactor with a liquid medium R3 (Tablle 1).

Statistical analyses

The results were analyzed with the commercial spreadsheet application Microsoft Excel, version 2010. The statistical method used was two sample t-tests to make pairwise comparisons between liquid and solid media. The tests were performed on the significance level $p < 0.05$.

Results

After four weeks on propagation, medium Pr plants looked as shown in Figure 2. Significant differences were observed between TIS and solid medium for both cultures. In solid medium strawberry plants multiplication ratio was 1.9 while in liquid – 4.1. Respectively for raspberry this ratio was 3.2 and 6.7 (Figure 3).

In strawberry cv. Tudla fresh and dry mass of TIS plants slight rise, which, however, was not statistically significant. This result was observed both in the period of propagation and rooting stage (Figure 4). There is no big deferens in shoots size (Figure 5a).

In raspberry increasing of fresh and dry weight was significant in TIS ($p \leq 0.05$), which applies to propagation and first rooting stage, but in plants grown on solid medium was observed a much higher percentage of dry matter as compared with those grown in the liquid medium. This means that the high fresh weight of the plants in a liquid medium is primarily due to water accumulation (Figure 6). Plants appear noticeably higher with larger leaves, but also partially hyperhydrated (Figure 5b).

Root initiation of strawberry began about 3 to 5 weeks

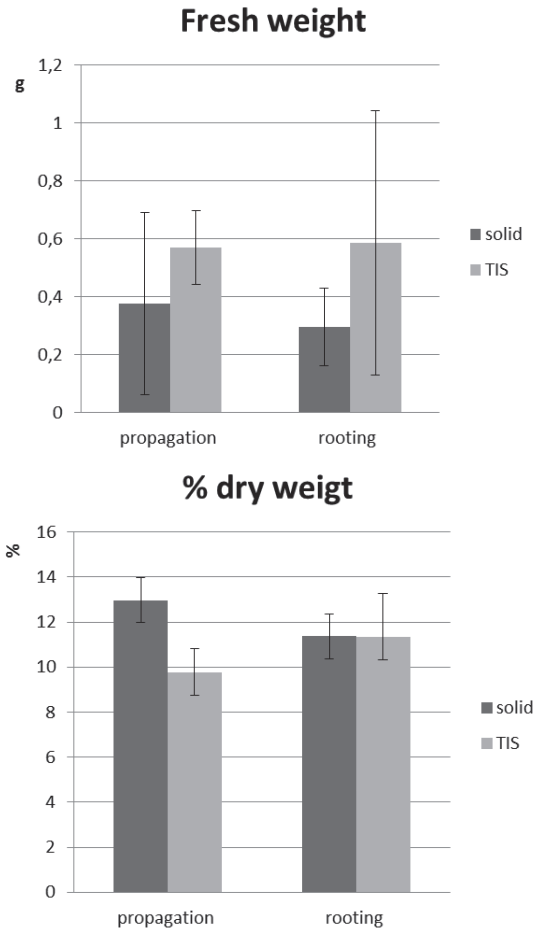

Fig. 4. Biomass accumulation for strawberry plants cv. Polka (Error bars indicate standard deviation. Right graphic is percent dry from fresh weight)

after transferring on rooting medium. All plants were rooted successfully in both liquid and solid medium.

For the same time only few raspberry plants formed roots in the same condition. In addition, many plants (about 50%) showed hyperhydric symptoms (translucent, brittle stems and leaves) on liquid medium.

That is the reason we tested another 3 variants of rooting systems mentioned in materials and methods.

All plants of the three variants are rooted successful within 4 weeks. They had no problem with hyperhydration. All variants showed relatively high percent dry weight, where the plants transferred on solid medium from liquid one (variant 2) was highest (Figure 7). Rooted plants were successfully adapted in soil in a greenhouse.

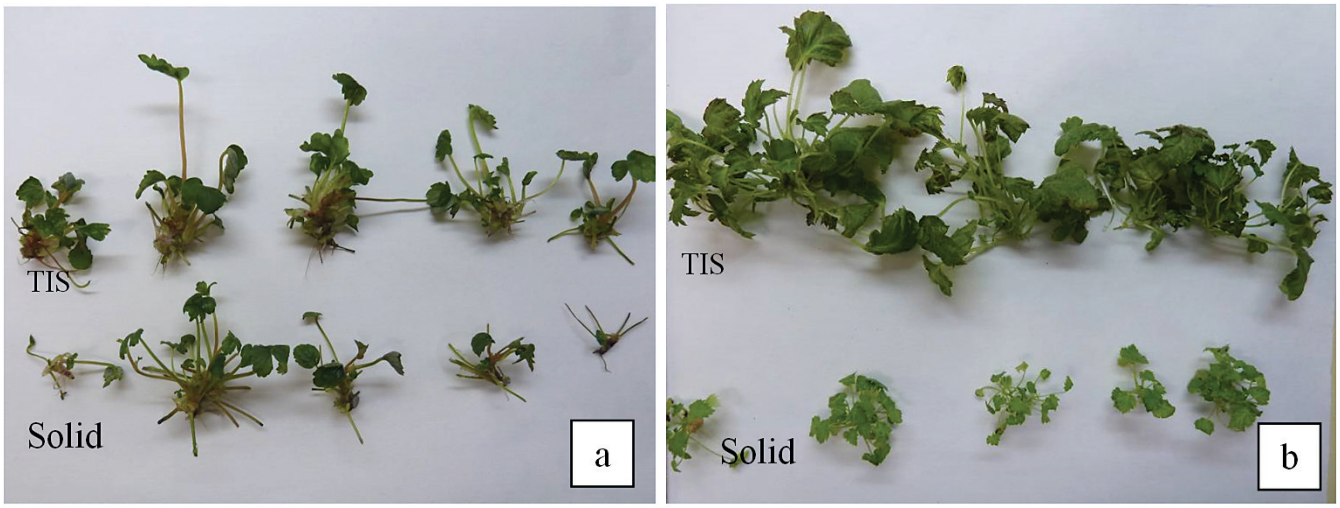

Fig. 5. Experimental plants after 4 weeks cultivation on Pr medium (Table 1). *a* – strawberry, *b* – raspberry

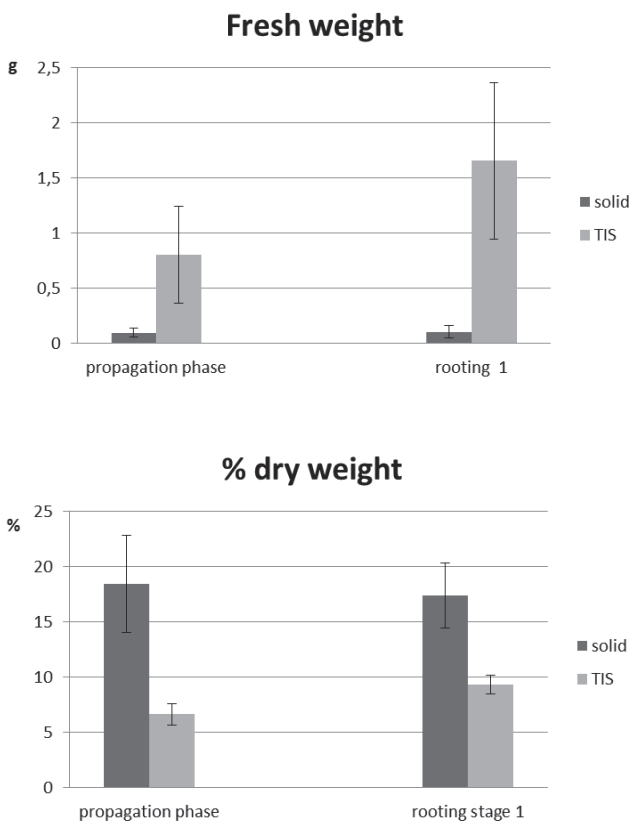

Fig. 6. Biomass accumulation of raspberry during the propagation and first rooting stage

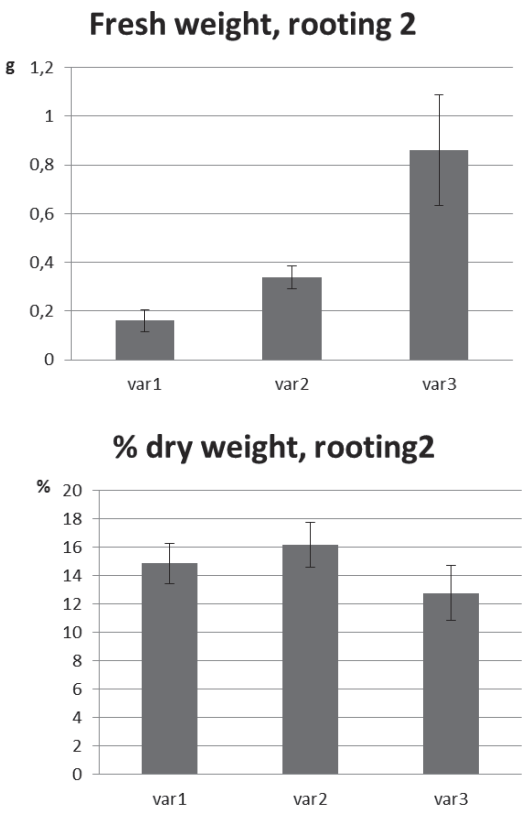

Fig. 7. Biomass accumulation for raspberry during the second rooting stage. Var. 1, 2 and 3 are described in materials and methods (Error bars indicate standard deviation)

Discussion

Results that we received for shoot multiplication of raspberry are different in comparison with those of Welander and colleagues (2014). They were observed no significant differences between TIS and agar medium for either total number of shoots or number of ready for rooting shoots for *Digitalis* and *Rubus*. In *Siraitia grosvenorii*, it was revealed that shoot multiplication rate and shoot length were significantly better in TIS than on solid and liquid media (Yan et al., 2010; Zhu et al. 2005) also concluded higher multiplication rate (apple rootstock M26) in TIS compared with solid medium. In a study on pineapple (*Ananas comosus* L. Merr), the shoot number was higher for TIS than for solid or other liquid medium (Escalona et al., 1999). However, Damiano et al. (2005) compared TIS and solid medium on apple (Jork 9), peach (cv. Yumyeong), cherry (cv. Biggareau Burlat) and plum (cv. Adara) and found no difference in multiplication rate between TIS and solid medium.

It seems that there is species and varieties dependency, but in most cases cultivation in TIS leads to increase multiplication rate.

The increase in dry weight was closely related to cell division and new material synthesis (Sunderland, 1960). Welander et al. (2014) proved that *Digitalis* and *Echinacea* gained significantly more weight during cultivation in TIS compared to agar medium, while *Rubus* showed the opposite.

This is also in contrast to our results.

Conclusions

TIS is a promising alternative system of mass propagation for strawberries and raspberries. The behavior of different species (strawberry and raspberry) on liquid medium propagation depends of the genotype.

Growing plants in a bioreactor is appropriate if the goal is rapid accumulation of fresh biomass.

The different purposes of application of the bioreactor system requires further optimization of the growing medium and precise conditions of cultivation of each species and variety.

Multiplication and rooting protocol for strawberries could be entirely in liquid medium, because strawberries are adaptable to growing conditions and tolerant of hyperhydration and they rooted for a shorter period.

The problem of hyperhydration in raspberry occurred when the plants stay more time in liquid medium. Eliminating this factor, we suggest our protocol for mass propagation of raspberry

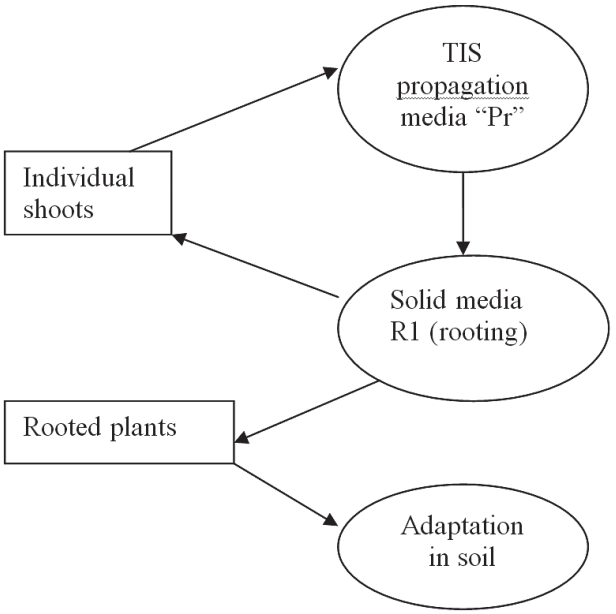

Fig. 8. Protocol for mass propagation of raspberry

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