Turkish Journal of Agriculture - Food Science and Technology, 7(2): 214 - 219, 2019
DOI: https://doi.org/10.24925/turjaf.v7i2.214-219.2202

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology

Use an Organic Biostimulant (Vermicompost Tea) For Enhancement *In Vıtro* Callus Growth in Sainfoin (*Onobrychis viciifolia* Scop.)

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ARTICLE INFO

Research Article

Received : 02/09/2018
Accepted : 23/01/2019

Keywords:
Sainfoin
Vermicompost Tea
Callus growth
Image Analysis
NI Vision Assistant module

ABSTRACT

The health and vitality of callus growth is one of the prerequisites for the success of further *in vitro* studies. This study investigated the efficiency of different percentage (0%, 10%, 20%, 30%, and 40%) of vermicompost tea as an organic substance on *in vitro* callus growth in sainfoin. Morpho-physiological responses of calli to vermicompost tea measured under *in vitro* conditions. As a result of this investigation, a combination of plant growth regulators (4 mg/l BAP and 0.5 mg/l NAA) with 20% of vermicompost tea causing significant callus initiation and growth in sainfoin stem explants. Under the light of these scientific findings, vermicompost tea might be used as an organic bio stimulant for efficient callus growth and complementary to commercial chemical hormones in sainfoin. This research is important due to it can contribute positively to the plant species that are difficult in terms of callus growth and plant regeneration in tissue culture.

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Introduction

Sainfoin (*Onobrychis viciifolia*) is a perennial forage legume with great potential for use in sustainable agriculture due to its low input requirements, good drought tolerance, and production of forage rich in polyphenolic compounds, which are beneficial for animal health (Kempft et al., 2016). A total of approximately 170 Onobrychis species exists throughout the world mainly in southwestern Asia, Mediterranean region, and temperate Europe and Asia. Of 55 Onobrychis species, 28 are endemic to Turkey (Uzun et al., 2017).

Vermicomposting is a non-thermophilic, boioxidative process that involves earthworms and associated microbes. This biological organic waste decomposition process yields the biofertilizer namely the vermicompost (Pathma and Sakthivel, 2012). Vermicompost produced by the activity of earthworms is rich in macro and micronutrients, vitamins, growth hormones, enzymes such as proteases, amylases, lipase, cellulase and chitinase and immobilized microflora (Kashyap et al., 2017). Earthworms promote the production of plant hormones auxins, gibberellins and cytokinins from organic waste. Auxins are responsible for cell elongation, cytokinins for promoting cell division and gibberellins for stem elongation. These hormones are dose significant and play a fundamental role in plant metabolism. They can influence plant growth and development as well as crop quality significantly when present at very low concentrations (Kashyap et al., 2013).

Slight changes in colour or geometric features of a callus can easily be overlooked by eye inspection, however the technique of image processing is a high speed automatic method with high magnification which provides better accuracy to calculate subtle visible *in vitro* data (Mansouri et al., 2015). Accurately measuring cell growth is an important aspect of most plant cell culture experiments. Callus growth may also be monitored qualitatively (by visually rating increases in colony size, for example) or indirectly (by measuring changes in the total weight of the culture dish), but these methods can be subjective, vulnerable to bias, and incapable of detecting small changes (James et al., 2004). Micro computerized image analysis (machine vision) is a technique which has particular merit for evaluation of plant microcultures, since it permits direct, objective, non-intrusive visualization and measurement of both cell and differentiated cultures. It capitalizes on each of the advantages of visual observation with the human eye, yet provides a concrete, quantitative, unbiased appraisal of culture performance. As a research device, image analysis provides a far more satisfying way to assess subtle visible *in vitro* data (Simith, 1995).

Plant tissue culture is the one of the most powerful tools for induction of fast crop improvements in modern plant breeding age (Kumlay and Ercisli, 2015). However, efficient plant regeneration from cultured cells and tissues is required for the successful application of biotechnology in crop improvement. Therefore, the success of cell and tissue culture research depends upon reliable callus culture and plant regeneration procedures. For sainfoin (*O. viciifolia* Scop.) and its species, many researchers reported effective protocol for callus initiation, grown and *in vitro* regeneration (Tamas and Savatti ,2006; Çeliktaş et al., 2006; Karamian and Ranjbar, 2006; Saglam, 2010; Mohajer et al., 2012; Kamalvand and Karamian, 2013; Okcu and Şengül, 2014; Yıldız and Ekiz, 2014; Honarmand et al., 2016). In these studies, combinations of commercial chemicals (phytohormones) with different concentrations were used. All these chemicals are very expensive and also some of them have toxic effects depend on their concentrations in plant cell or tissue. Therefore, this study was conducted to investigate the contribution of vermicompost tea as an organic bio stimulant on callus growth in sainfoin. To our best knowledge, this is the first report to determine the effect of vermicompost tea as an organic bio stimulant on callus growth in sainfoin.

Materials and Methods

Seed Sterilization and Germination

Dehulled seeds of sainfoin (*O. viciifolia* Scop.) were surface sterilized using 20% of commercial bleach (ACE, containing 5% sodium hypochlorite) for 20 min with continuous stirring and were then rinsed three times with sterile water. Sterilized seeds were germinated on a basal medium of Murashige and Skoog's (MS) mineral salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, and 0.65% agar. All cultures were incubated at 25±1°C under cool white fluorescent light (about 2,000 lux) with a 16 h light/8 h dark photoperiod. The pH of the medium was adjusted to 5.6-5.8 prior to autoclaving.

Callus Induction and Growth

Stems was used as explants. Stem explants were taken from 30-day seedling of sainfoin. The stem explants were cultivated on MS medium containing 4 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l α–naphthaleneacetic acid (NAA) (Çöçü, 2008) and different percentages (10%, 20%, 30%, and 40%) of vermicompost tea for 4 weeks. Hormone MS medium was used as a control. The vermicompost tea were sterilized by filter sterilization (0.22 µm) and added into 200 ml MS medium after autoclaving to protect biological properties of tea. 1.5 ml of tea were added for each treatment, except control.

Image Processing Procedure

The maximum length, maximum width, projection area values evaluated from callus samples (Figure 1) by using LabVIEW software. Beyaz et al. (2017) used this software for the measurements of biological material dimensions. Also, this dimensions and projection areas had been detached by this software in his other studies (Beyaz, 2016; Beyaz, 2018). Additionally, mean Red, mean Green, mean Blue colour values evaluated from observed projection areas. For this aim, ten callus samples were placed in each of petri dishes. Four petri dishes were used for each dosage of Vermicompost tea applications. Four different percentage of Vermicompost were used for laboratory experiments. Also, a group of callus samples was used as witness samples. 200 callus samples were observed and evaluated from 20 images. The resolution of the images was 4128 x 3096 with 72 dpi. A callus sample image measurement in NI Vision Assistant module can be seen in Figure 21200 data were evaluated as pixel values and saved in Microsoft Excel files. Also, a ruler was used to get real dimensions of the callus samples as calibration plate on images turn pixel values to the metric unit. The mean values of this data were evaluated in Microsoft Excel software and accepted as the last results.

Physiological Observations:

Relative water content (RWC)

Relative Water Content (RWC) of callus was calculated by using the formula (Kishor, 1999):

$$\text{RWC}(\%) = \frac{\text{Fresh wt}}{(\text{Fresh wt} - \text{Dry wt})} \times 100$$

Chlorophyll Contents

Chlorophyll contents was estimated by Curtis and Shetty (1996) and is expressed "µg chlorophyll/g of fresh tissue". According to this protocol, 50 mg of callus sample was put into 3 ml of methanol and kept at 23°C in darkness for 2 hours to allow the chlorophyll in the green material to dissolve into the methanol. Then, the optic density (OD) of 1.5 ml of the liquid part (the chlorophyll-containing methanol) was determined at 650 and 665 nm using spectrophotometry.

Preparing Vermicompost Tea Stock

The Vermicompost tea was prepared determined protocol by Edwars et al. (Edwards et al., 2010). According to this protocol, Daily 1:20 and 1:10 solid vermicompost: water solutions (without fermentation) will be shaken for 4 hours with the help of a ventilation motor and mechanical stirrer and applied in spray form by extraction. Solid vermicompost raw material is cow manure. The main stock prepares as 40%. 30%, 20% and 10% proportion of vermicompost tea were prepared from main stock (40%). The final volume of 30%, 20% and 10% proportion is 50 ml.

Statistical Analysis

Four replicates were tested, and there were ten explants per replication. Data were statistically analysed by Duncan's multiple range test using IBM SPSS (Version 21.) for Windows. Data given in percentages were subjected to arcsine (√X) transformation before statistical analysis (Snedor and Cochran, 1968).

Results

The effect of different levels (0%, 10%, 20%, 30%, and 40%) of vermicompost tea (VT) on morpho-physiological responses of calli were shown in Table 1. and Table 2. In terms of callus percentage, callus fresh and dry weights and relative water contents (RWC), the highest values (100%, 6.60 g, 0.58 g, 91.16%, respectively) were recorded at 20% of VT. The lowest values according to callus percentage,

callus fresh and dry weights and relative water contents (RWC) were determined in the control group (0% VT). The texture of calli in all treatments show as compact. According to the chlorophyll contents (chl. a, chl. b, and total), 20% of VT treatment showed an induced effect on calli of sainfoin. Chlorophyll a, b and total were 37.10, 21.30 and 38.57 µg chlorophyll/g of fresh tissue, respectively (Table 1.). However, the lowest values of chlorophyll contents were observed at treatment of %10 of vermicompost tea. In the present study, the max length of calli, max width of calli and area of calli was achieved on MS supplemented with 20% of vermicompost tea. Callus max. length, callus max. width and callus area were 1.98 cm, 1.35 cm and 2.65 cm^2, respectively (Table 2.). The treatments of %20 of vermicompost tea clearly trigger growth of calli (Figure 1.). However, it was found that there was no significant effect of different levels of VT (%10, %30, and %40) on these growth parameters. The lowest RGB (red, green and blue) values, except G, were obtained from calli, cultivated in MS medium supplemented with 20% of VT. On the other hand, the highest values, except B, were recorded in treatments of %40 of vermicompost tea (Table 2).

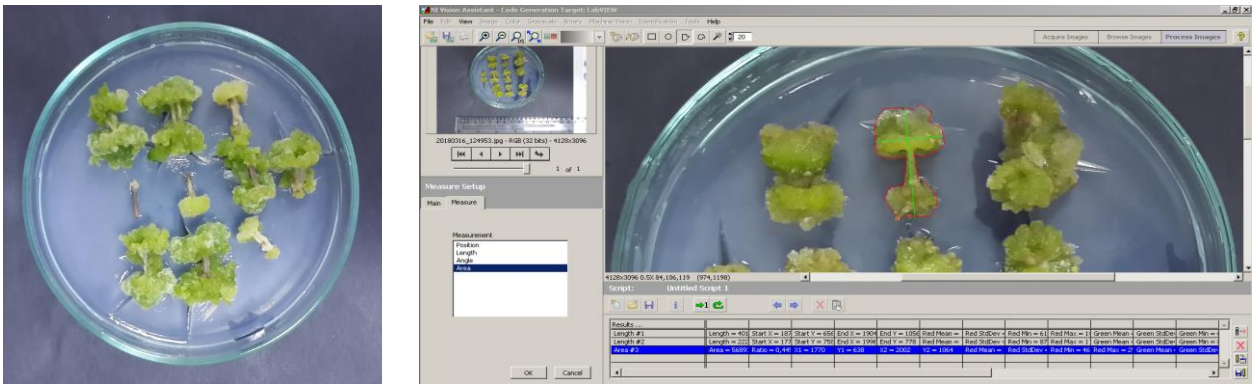

Figure 1 Callus samples in petri dishes and samples image measurements step in NI Vision Asistant module.

Table 1 Morpho-physiological responses of callus to different percentage of vermicompost tea (VT)

Treatments	Callus percentage (%) ns	Fresh wt (g) **	Dry wt (g) *	RWC (%) **	Texture	Chlorophyll (µg chlorophyll/g of fresh tissue) **		
						a	b	total
PGR+0% VT	95.0	4.33^{b}	0.47^{c}	89.05^{ab}	Compact	20.68^{ab}	24.82^{a}	33.97^{ab}
PGR+10% VT	95.0	5.19^{b}	0.49^{c}	90.53^{b}	Compact	17.90^{ab}	11.96^{b}	20.24^{c}
PGR+20% VT	100.0	6.60^{a}	0.58^{ab}	91.16^{a}	Compact	37.10^{a}	21.30^{a}	38.57^{a}
PGR+30% VT	90.0	4.73^{b}	0.50^{abc}	89.09^{b}	Compact	15.18^{c}	13.09^{b}	19.99^{c}
PGR+40% VT	97.5	6.27^{a}	0.59^{a}	90.47^{ab}	Compact	25.64^{b}	17.33^{ab}	29.17^{b}

**: P<0.01, *: P<0.05 and ns: non-significant and PGR: Plant Growt Regulator

Table 2 Geometric features and colors of a callus samples under different pertancage of vermicompost tea (VT)

Treatments	Max. Length (cm) ns	Max. Width (cm) ns	Area (cm^2)**	Mean Red ns	Mean Green ns	Mean Blue ns
PGR+0% VT	1.90	1.00	1.48 b	147	149	94
PGR+10% VT	1.71	1.05	1.58 b	156	159	94
PGR+20% VT	1.98	1.35	2.65 a	141	151	80
PGR+30% VT	1.75	1.02	1.58 b	153	156	100
PGR+40% VT	1.84	1.08	1.79 b	154	157	95

**: P<0.01, *: P<0.05, ns: non-significant and PGR: Plant Growt Regulator

Discussion

Exogenous application of commercially produced chemical hormones (auxin and cytokinin) have been widely used to generate callus in various plant species (Ikeuchi et al., 2013). Differently, in the present study, the effect or contribution of vermicompost tea as an organic bio stimulant on callus growth and development were investigated. The amounts of growth of the callus pieces were examined after four weeks incubation (Table 1., Figure 1.). Well-developed callus is a desirable prerequisite for plant regeneration because callus offers the greatest opportunity for *in vitro* selection and production of genetic variations. Our results indicated that application of VT with the PGRs (4 mg/l BAP and 0.5 mg/l NAA) to callus growth and development give good offerings in sainfoin. The growth parameters (fresh weight, dry weight and relative water content) were significantly (P<0.01) increased with increasing levels of vermicompost tea. However, the findings indicated that dosage of vermicompost tea is important and there is a threshold of dosage of VT for a positive effect on growth parameters.

In this present study, the dosage threshold of VT determined as %20 for growth parameters of calli. Similarly, Morales-Corts et al. (2018), reported that there is a positive effect of vermicompost tea on tomato growth and is also a dosage threshold. On the other hand, The results of this study were agreement the findings of Hargreaves et al. (2009) and Marín et al. (2014).

Chlorophyll, which traps light and transfers energy for driving photochemical reactions, is one of the most photochemically active compounds in photosynthesis (Yadav et al., 2010). Callus tissue cells derived from some plants also contain chlorophyll with similar activities, by which callus cells should grow autotrophically (Fukami and Hildebrandt, 1967). The results of our study indicated that vermicompost tea clearly enhance the chlorophyll contents of calli. Photosynthetic efficiency and cell growth associated with quantification of chlorophyll contents have been well illustrated by Masojidek et al. (2000) and Tremblin et al. (2000). The increase in cell growth was attributed to higher total chlorophyll levels (Fujimoto et al., 2012). Obtained green healthy calli as a starting materials are imported to formation of healthy shoots in indirect organogenesis. Photosynthetic activity as chlorophyll a, chlorophyll b and total chlorophyll content which was an indicator of plant health and vitality and was also marker for plant health and vitality of callus. The results of present study indicated that 20% of vermicompost tea can contribute to chlorophyll contents of calli. So it can be concluded that vermicompost tea can be use up to a certain threshold (20%) to obtain healthy and well development calli in sainfoin. In terms of health and vitality of green material, the highest quantity of chlorophyll contents is a good indicator at the level ranging from the whole plant, the leaf or cell, to explant.

The image analysis technique is a flexible and statistically powerful tool for monitoring plant cell cultures. The growth of callus can be monitored effectively and non-invasively (James, 2004). Mansouri et al. (2015), reported that image analysis can be used to measure morpho-physiological parameters of callus. In the present study, callus growth parameters or geometric features such as callus max length, max width and area were measured by using image analysis techniques. We observed that the effect of vermicompost tea on the geometric features of calli are varied. It seem that there is no significant effect of different dosage (%10, %20 and %30) of vermicompost tea on the geometric features, except callus area, of calli, except %20. On the other hands we found that area of callus significantly ($P<0.01$) effected by vermicompost tea. The findings of present study show that vermicompost tea in regeneration medium is directly effect callus growth in a positive ways up to a specific thereshold (20%). These positive attributes might be sourced from some compenents, which have the hormone-like activity, of the vermicompost. Similar results reported by Kashyap et al. (2013) and Kashyap et al. (2017) in different plant species.

In recent years, several image analysis methods have been developed to monitor various parameters of plant health status and cell growth using red, green and blue color channels (RGB). In the present study, RGB colours use to determine health status and growth of calli which were exposed to different percentage of vermicompost tea (VT). Colour images were captured with a digital camera, processed in MATLAB, and various RGB parameters were determined (Table 2.). Although the RGB values are not statistically important, interestingly, the low values (141, 151 and 80) of RGB (except, G) obtained from well-developed calli which are exposed to 20% of VT. The RGB parameters are generally use for determine chlorophyll contents of leaves based-on colours in plants (Yadav et al., 2010). Higher plants contain primarily two types of chlorophylls, namely chlorophyll a and chlorophyll b. Chlorophyll a is bluish green and chlorophyll b is yellowish green (Palta, 1990). Quantification of chlorophyll a associated with photosynthetic efficiency and cell growth (Masojidek et al., 2000; Tremblin et al., 2000). The findings show that the highest content of chl a (37.10 µg chlorophyll/g of fresh tissue) obtained from calli exposed to 20% of VT However, the lowest value (80) of B color, which related with chl a, was recorded at 20% of VT application. On the other hand, when observe the other application (10%, 30%, and 40%) in Table 2. From to these results, it might inference that B take a lower value when the chlorophyll a content is high. Yadav et al. (2010), reported that there is a negative correlation between R and G with the chlorophyll content meanwhile there is a positive correlation with B chromate. The results of current study indicated that low R and G values obtained from calli which have highest total chlorophyll contents (38.57 µg chlorophyll/g of fresh tissue). So these results are similar with results of Yadav et al. (2010).

Overall, the mean value of maximum length (1.98 cm) and maximum width (1.35) with nearly 3 mm difference were observed in 20% of VT application. These values are critical in this kind of micro-level laboratory studies. Also, the mean value of the maximum area (2.65 cm^2) with the 1 cm^2 difference observed in 20% of vermicompost tea. Additionally, minimum mean red (141) and minimum mean blue (80) values observed in 20% of vermicompost tea. It assumed that these colours can be related to different colour pigments which are newly grown parts of callus samples. On the contrary of the other colour values mean green level (151) of the 20% of vermicompost tea was average between the other groups.

Conclusion

The results of present study show that there is a significant ($P<0.01$) contribution of 20% of vermicompost tea as an organic substance on callus growth of sainfoin. However, using only vermicompost tea with a different percentage (10%, 20%, 30% and 40%) in MS medium significantly effect callus growth from stem explants (Data not show) of sainfoin. On the other hand, more work is needed such as optimization of percentage of vermicompost tea to obtain health and vital callus. In plant tissue culture, many factors such as genotypes of plants, type of explants, concentrations and combination of plant growth regulators (especially this factor), culture conditions, medium type, carbon source and gelling agents, chemical composition and physical state of the culture medium effect callus growth and development. Therefore, application of vermicompost tea with all these factors can be work in the future to obtained well-developed callus and *in vitro* regeneration in sainfoin. On the other hand, we assume that the protocol presented in this study could be

used for other plants, that are recalcitrant to manipulation *in vitro*, to obtain highest callus growth and development *in vitro*.

Acknowledgement

The author thanks to Dr. Abdullah Beyaz (Ankara University, Department of Agricultural Machinery and Technologies Engineering, Turkey) for his valuable contributions in the image analysis process.

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