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GROWTH IMPROVEMENT OF *NICOTIANA* AND *ARABIDOPSIS* IN VITRO BY MICROALGAL CONDITIONED MEDIA

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Conditioned medium (CM) is a general term describing media in which cells have already been cultivated for some time. Such media, usually clarified by filtration, have been used by plant biotechnologists as additives supporting the growth of cell suspensions, organs and whole plants. This study examined the effect of CM obtained from green alga *Desmodesmus subspicatus* on the growth and functioning of the photosynthetic apparatus of *Nicotiana tabacum* and *Arabidopsis thaliana* in culture in vitro. Plants where cultured on CM diluted 1.25-, 2- and 5-fold with MS medium. The increase in fresh and dry weight was highest in tobacco and *Arabidopsis* cultured on CM/2 and CM/1.25 media. Those two concentrations also increased the amount of chlorophylls in both plants tested. CM improved parameter PI (reflecting the photosynthetic "vitality" of the organism) and electron transport efficiency, and increased the fraction of active reaction centers. Analysis of chlorophyll fluorescence in vivo suggests that the improvement of these plants grown in the presence of algal CM may result from stimulation of photosynthesis. Algal CM offers a convenient, cheap, universal supplement for stimulating the growth of higher plants in vitro.

Key words: Algal exudates, in vitro culture, growth improvement, photosynthesis.

INTRODUCTION

A great number of discoveries in the field of plant cell culture, tissue culture and genetic engineering have been made through research involving *Nicotiana tabacum* and *Arabidopsis thaliana* (Ganaphathi et al., 2004; Koornneef and Meinke, 2010). In recent years, plants have been found to be sources of recombinant protein or other economically interesting molecules such as pharmaceuticals or functional secondary metabolites (Boehm, 2007). Genetically modified *Nicotiana* and *Arabidopsis* cells are used to produce antibodies, vaccines and other therapeutic proteins (Koornneef and Meinke, 2010; Wiktorek-Smagur et al., 2012). Hence the need for low-cost methods of improving the growth and productivity of model plants such as tobacco or *Arabidopsis*.

The composition of the culture medium is one of the most important factors in successful cultivation of plant cells. A typical culture medium usually consists of sugars, vitamins and phytohormones, and in some cases organic additives such as coconut water, fruit juices, casein hydrolysate or yeast extract. A cell-free culture supernatant referred to as conditioned (or spent) medium (CM) is a special kind of additive. It contains a wide variety of organic compounds ranging from simple sugars or carboxylic acids to high molecular weight substances such as proteins and polysaccharides produced and secreted by cells to the culture medium (Wink, 1994). Some of these substances reveal biological activity able to stimulate growth (Schröder and Knoop, 1995; Matsubayashi and Sakagami, 1996) or to inhibit it (Kobayashi et al., 2000). Although undefined, CMs obtained from higher plants are often used in biotechnological applications to support the proliferation of many different plant cells or tissues. In some species such media are essential for successful regeneration of fertile plants from microspores (Patel et al., 2004; Sidhu and Davies, 2009) and somatic hybrids (Horita et al., 2003), promotion of somatic embryogenesis (Ben Amar et al., 2010; Couillerot et al., 2012) or recovery of cryopreserved cells (Hargreaves et al., 2002). Preparing CM from higher plant cultures may be troublesome and laborious, so green algae culture may offer a better source of conditioned medium. Many of these

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organisms occur naturally as unicells, have low nutrient requirements, rapidly proliferate, and may be cultured in mineral media. Like higher plants, they secrete organic compounds into their culture medium with self-stimulatory properties (Imaizumi et al., 2007; Grabski and Tukaj, 2008). Preparation of highly active conditioned medium from algal suspensions is relatively easy and cost-effective. Despite the potential usefulness of such media in biotechnology, there are few papers on the interaction of algal extracellular matter with terrestrial plant growth. Preliminary studies indicate that algal extracellular exudates are indeed able to stimulate the growth of plant cells, even in a phylogenetically distinct higher plant such as *Silene vulgaris* (Caryophyllaceae) (Hanus-Fajerska et al., 2009) or in *Daphne* species (Wiszniewska et al., 2013). Previous studies have examined the effects on higher plants of compound(s) released into medium by ubiquitously occurring phytoplankton in suspension cell cultures and organ or embryo cultures (Hanus-Fajerska et al., 2009; Shrivastava and Banerjee, 2009; Gurusaravanan et al., 2013). Our present work is the first demonstration of the effect of *Desmodesmus subspicatus*-conditioned medium on the growth of whole model plants (*Nicotiana tabacum*, *Arabidopsis thaliana*) cultured in vitro. Here we also consider the mechanism of this improvement.

MATERIALS AND METHODS

PLANT MATERIALS

Nicotiana tabacum var. Bursan and *Arabidopsis thaliana* var. Landsberg were the tested plants. Conditioned medium was obtained from the unicellular green alga *Desmodesmus subspicatus* (previously *Scenedesmus subspicatus*) (Chodat), strain 2594.

PREPARATION OF CONDITIONED MEDIUM (CM)

Desmodesmus subspicatus was precultured on liquid Bold Basal Medium (Bischoff and Bold, 1963) in 100 cm^3 Erlenmeyer flasks containing 50 cm^3 medium until the exponential growth phase. Then the algae were transferred to a 3 dm^3 flask containing fresh BBM. The initial cell density was 0.4×10^6 cells cm^{-3} and the final volume of suspension was 2.5 dm^3. The pH of the medium at the start of the culture was adjusted to 6.9±0.1. The flasks were illuminated from one side by cool fluorescent tubes (Philips TLD 54W/94) supplying 160 μmol (photons) $\times m^{-2} \times s^{-1}$ photosynthetically active radiation (PAR, 380–690 nm) measured at the culture vessel surface. The cultures were continuously bubbled with atmospheric air passed through a bacteriological filter (Sartorius 2000, 0.2 mm PTFE). *Desmodesmus subspicatus* was grown at 26±2°C. After 7 days of culture the algal cells were removed from the medium by centrifugation at 1,000 × g for 15 min. The supernatant was filtered through a membrane filter (0.2 μm, Sartorius, Germany). Undiluted CM filtrate was stored in darkness at 4°C. It was diluted 1.25-, 2- and 5-fold (CM/1.25, CM/2, CM/5) with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

CULTURE

Seeds of *Nicotiana* and *Arabidopsis* were enclosed in Whatmann 3MM paper. These were surface-disinfected with 70% alcohol for 5–10 s and with 20% (v/v) Ace bleach (2% sodium hypochlorite) with 1% TritonX-100 for 10 min, followed by three rinses with sterilized deionized water. Seeds were placed in 200 cm^3-capacity glass jars for plant tissue culture, containing Murashige and Skoog medium. MS was prepared as follows: 4.4 g powdered medium (Murashige and Skoog basal medium with Gamborg's vitamins, SIGMA) was dissolved in 900 cm^3 deionized water, followed by addition of sucrose (3%; w/v) and agar (0.9%; w/v), adjustment to pH 5.8 with 1 mol × dm^{-3} NaOH, and supplementation with water to a final volume of 1 dm^3. The medium was autoclaved at 121°C for 20 min. Germinating seeds were placed in a culture chamber under constant light intensity of 50–60 μmol × m^{-2} × s^{-1} at 22±2°C for 2–3 weeks, then transferred to glass jars containing the same media and kept under the same light and temperature conditions to allow further growth. After 4–5 weeks, explants were propagated in sterile conditions.

To assess the effect of conditioned medium on the growth of *N. tabacum* and *A. thaliana* in culture in vitro, plants were grown on CM diluted 1.25-, 2- and 5-fold with MS and on non-CM MS medium (control). All of the medium variants contained the same quantity of powdered MS medium, sucrose (3%; w/v) and agar (0.9%; w/v), and different volumes of water and undiluted conditioned medium. The pH of each medium was adjusted to 5.8 using 1 mol × dm^{-3} NaOH. Explants were prepared from 4–5-week-old plants. Leaves were removed from the tobacco stems, which were then cut into single node segments. Two of these explants were placed in one vessel for plant tissue culture. *Arabidopsis* plants were propagated by separating adventitious rosettes. Four *Arabidopsis* explants were placed in one glass jar. There were three replicates of all variants. The plants were grown in a phytotron under strictly controlled conditions for three (*N. tabacum*) or four weeks (*A. thaliana*) under continuous cool fluorescent light (50 μmol × m^{-2} × s^{-1}) at 22±2°C. The observations and measurements included plant morphology, fresh and dry weight of plant material, content of photosynthetic pigments, and chlorophyll *a* fluorescence.

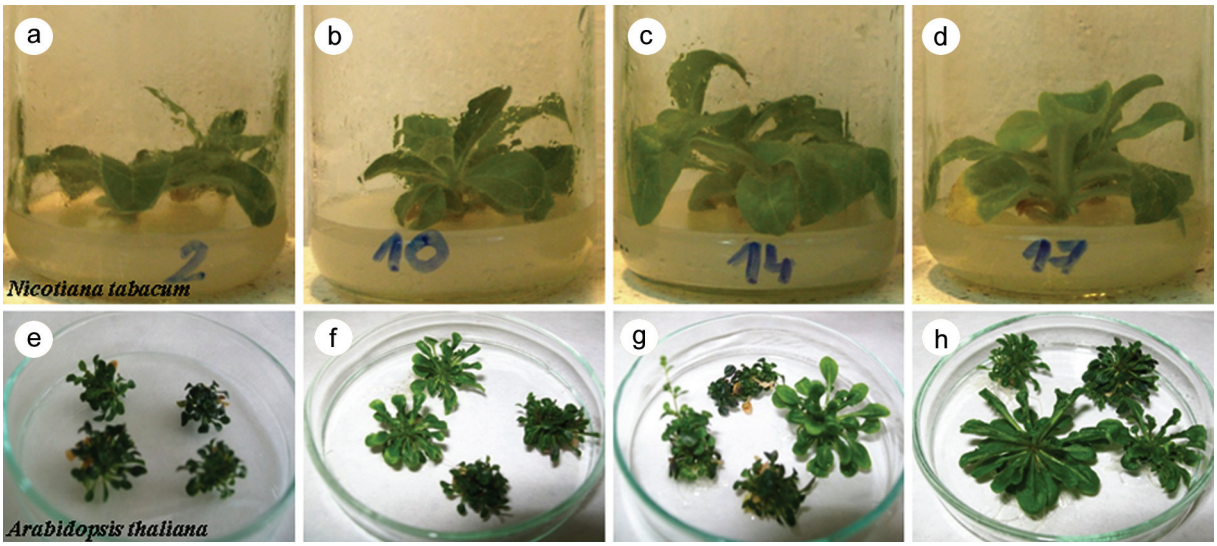

Fig. 1. *Nicotiana tabacum* after 3 weeks of growth and *Arabidopsis thaliana* after 4 weeks of growth on control medium MS (**a, e**) and on algal-conditioned media at different dilutions: CM/5 (**b, f**), CM/2 (**c, g**), CM/1.25 (**d, h**).

FRESH AND DRY WEIGHT

After 3 or 4 weeks of culture the plants were removed from the plant tissue culture vessels. The roots of each plant were rinsed with distilled water to remove remnants of medium, then gently dried with a paper towel, and whole plants were weighed on an analytical balance. After measuring fresh weight the plants were dried separately at 105°C to constant weight.

PHOTOSYNTHETIC PIGMENTS

Pigments were extracted from leaves of *Nicotiana* and *Arabidopsis*: 10 cm^3 100% acetone and a small amount of quartz sand was added to samples weighing 0.1 g. The plant material was mechanically disintegrated by 3-min homogenization with a vortex mixer. Then the homogenate was centrifuged for 10 min at 3000 × g. The absorbance of the acetone extract was spectrophotometrically measured in a 1 cm glass cuvette for wavelengths 664, 647 and 740 nm. The concentration of pigments was calculated according to the equations given by Lichtenthaler and Wellburn (1983), correcting for the turbidity of the sample (absorbance at 740 nm). Chlorophyll *a* and *b* content was then converted per gram fresh weight of *N. tabacum* and *Arabidopsis thaliana* leaves.

FLUORESCENCE MEASUREMENTS

Chlorophyll *a* fluorescence induction transients were measured at room temperature with a high-resolution Handy-PEA fluorimeter (Hansatech Ltd., Norfolk, UK). Before each measurement a shutter blade was clipped on leaves of the test plants. The samples were adapted for 30 min in darkness to complete re-oxidation of PS II electron acceptor molecules. During adaptation the shutter blade was closed. Then the shutter blades were opened and fluorescence induction was measured during 0.1 s red irradiation (650 nm) at 3000 μmol × m^{-2} × s^{-1}. The initial fluorescence value (F_0) was determined during the first 50 μs of measurement of the dark-adapted sample. The fluorescence signal was detected in intervals of 10 μs. The original data stored during measurements were used to calculate further parameters. The performance index (PI) was calculated according to Strasser et al. (1999). The maximum quantum yield of primary photochemistry (φ_{Po}), the efficiency with which a trapped exciton can move an electron further than the Q_A quinone along the electron transport chain (ψ_0), the probability that an absorbed photon will move an electron into the electron transport chain (φ_{Eo}), and the fraction of active reaction centers in the dark-adapted samples (RC/CS_0) were calculated according to Strasser et al. (2000).

STATISTICAL ANALYSIS

Statistical analyses employed MS Excel 2000 (Microsoft). The values given are means ±SE. Student's t test was used to test the significance of differences among the controls and treatments at $\alpha = 0.05$.

RESULTS AND DISCUSSION

CM added to the medium affected both the weight and morphology of the studied plants. Plants grown on CM diluted 1.25- and 2-fold were significantly

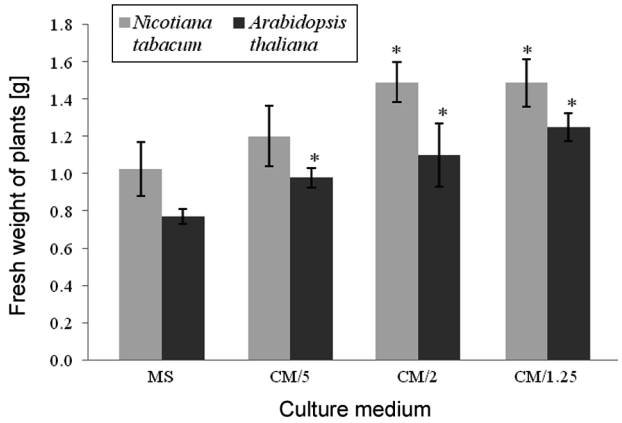

Fig. 2. Fresh weight of whole *Nicotiana tabacum* and *Arabidopsis thaliana* plants cultured on algal-conditioned medium (CM) diluted 1.25-, 2- and 5-fold (CM/1.25, CM/2, CM/5) and on MS (control). Error bars represent SE of means (n=8). Asterisks indicate significant differences between the respective control and CM-treated plants (t-test, a=0.05).

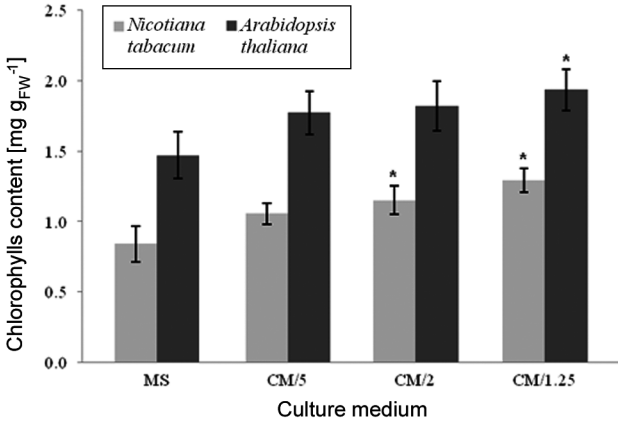

Fig. 4. Chlorophyll content in *Nicotiana tabacum* and *Arabidopsis thaliana* cultured on algal-conditioned medium (CM) diluted 1.25-, 2- and 5-fold (CM/1.25, CM/2, CM/5) and on MS (control). Error bars represent SE of means (n=6). Asterisks indicate significant differences between the respective control and CM-treated plants (t-test, a=0.05).

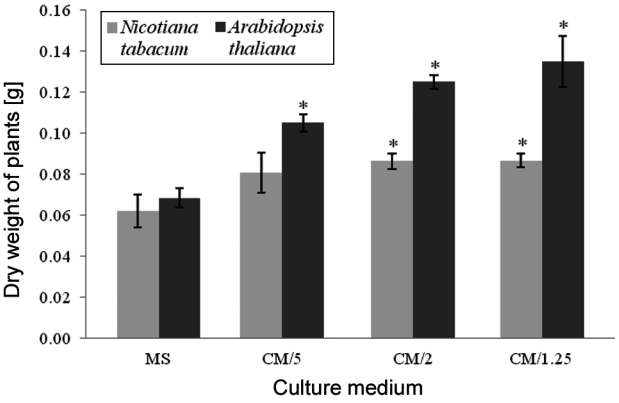

Fig. 3. Dry weight of whole *Nicotiana tabacum* and *Arabidopsis thaliana* plants cultured on algal-conditioned medium (CM) diluted 1.25-, 2- and 5-fold (CM/1.25, CM/2, CM/5) and on MS (control). Error bars represent SE of means (n=6). Asterisks indicate significant differences between the respective control and CM-treated plants (t-test, a=0.05).

bigger than those grown on the other tested media (Fig. 1). Fresh weight was significantly higher in tobacco cultured on CM/2 and CM/1.25 (145% of control, Fig. 2). Conditioned medium stimulated the growth of *Arabidopsis*: the mean fresh weight of plants grown on CM diluted 1.25-, 2- and 5-fold was significantly higher than that of plants grown on the control medium (MS). The increase of fresh weight was highest for *Arabidopsis* cultured on CM/1.25 (162% of control), and lower for plants grown on CM/2 and CM/5 (143% and 127% of control respectively). Both tested species showed higher dry weight in all CM treatments, but the effect was more pronounced in *A. thaliana* (Fig. 3), highest in the plants grown on CM/1.25 (198% of control). *Arabidopsis* grown on CM/2 and CM/5 showed significant increases of dry weight (183% and 154% of control). CM affected the dry weight of *N. tabacum* less (CM/1.25: 139% of control; CM/2: 140% of control).

Media conditioned using cultures of *Desmodesmus subspicatus* stimulated the growth of *Nicotiana* and *Arabidopsis* in culture in vitro. Such a protocol for in vitro propagation offers an ecofriendly and cheaper technology for use with many important plants. Previously we demonstrated the autoproliferating properties of CM/2 obtained from *Desmodesmus* (Grabski and Tukaj, 2008). The same CM diluted four times stimulated the growth of *Silene vulgaris* cells in suspension culture (Hanus-Fajerska et al., 2009). Depending on the dilution, algae-conditioned medium stimulates the growth, mitotic activity and biomass production of higher plants. Conditioned media derived from algae or protoplast culture are usually diluted 2- to 5-fold (Folling et al., 1995; Zhou et al., 2005). Undiluted CMs are thought to have an adverse effect on cell propagation due to the presence of toxic waste products and/or suboptimal composition, for example depleted nitrogen (Grabski and Tukaj, 2008).

The content of chlorophylls increased under the influence of conditioned medium. The stimulatory effect was highest (154% of control) in tobacco plants growing on CM/1.25 (Fig. 4). CM/2 medium also increased total chlorophylls in *N. tabacum* leaves (137% of control). The increase was less in

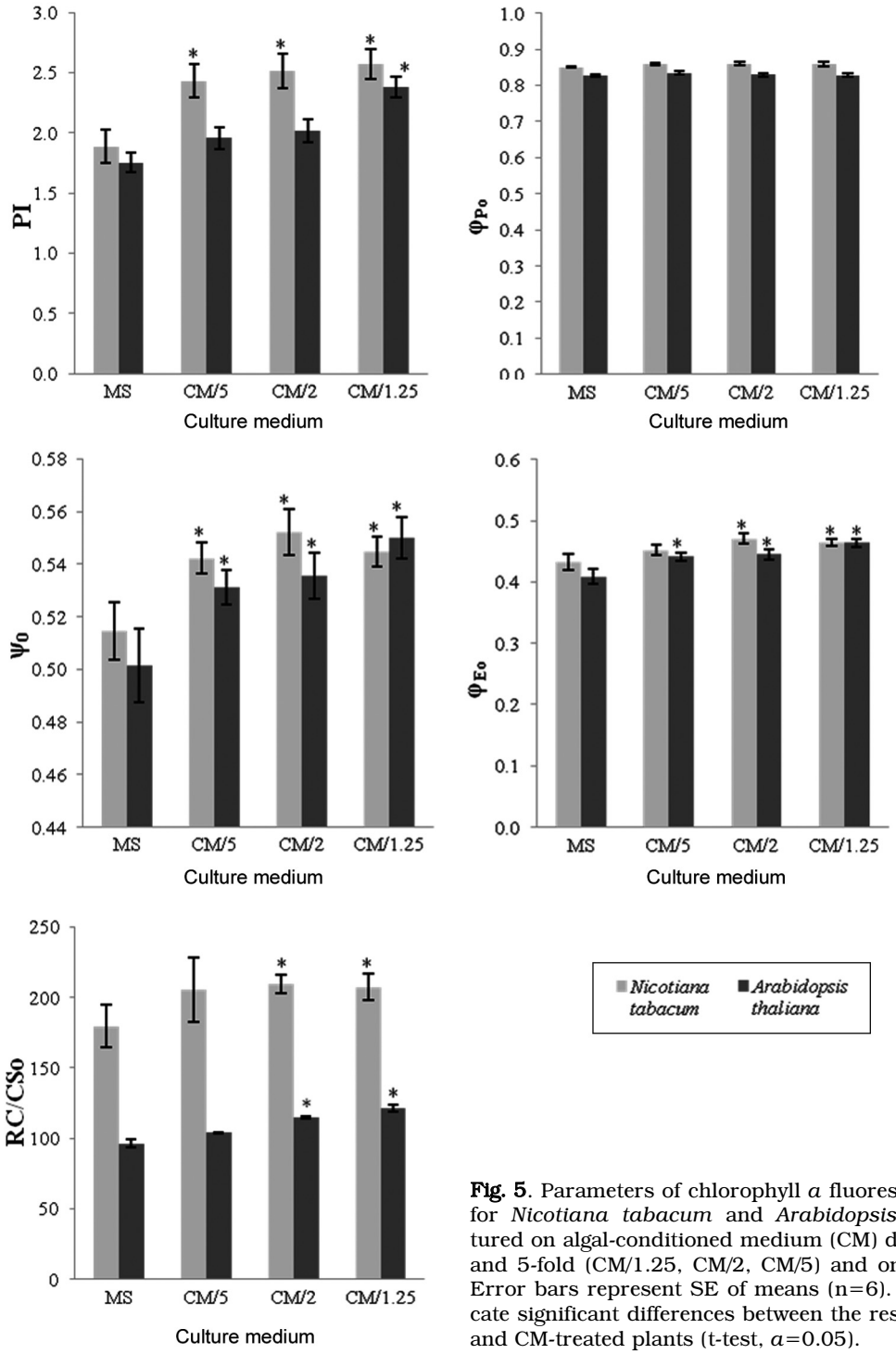

Fig. 5. Parameters of chlorophyll *a* fluorescence obtained for *Nicotiana tabacum* and *Arabidopsis thaliana* cultured on algal-conditioned medium (CM) diluted 1.25-, 2- and 5-fold (CM/1.25, CM/2, CM/5) and on MS (control). Error bars represent SE of means (n=6). Asterisks indicate significant differences between the respective control and CM-treated plants (t-test, α=0.05).

A. thaliana grown on CM/1.25 (123% of control). Spent media of *Aulosira fertilissima* also boosted chlorophyll *a* and *b* content in *Jatropha curcas*, accompanied by an increase of photosynthetic efficiency (Shrivastava and Banerjee, 2009).

To determine the reasons for growth improvement in CM-grown plants, we examined the functioning of the photosynthetic apparatus using OJIP technology. Based on analyses of the polyphasic chlorophyll *a* fluorescence induction curve we esti-

mated the performance index (PI), quantum efficiencies or flux ratios, phenomenological activity and density of active reaction centers per excited cross-section (Fig. 5) (Strasser et al., 1995). PI reflects the photosynthetic "vitality" of the organism and depends on the density of active reaction centers as well as on the efficiency of energy absorption, trapping or transfer beyond the Q_A quinone. The PI values were higher (~135% of control) in tobacco grown on CM/1.25 and CM/2, and in *Arabidopsis* grown on CM/1.25 (~120% of control). Photosynthetic apparatus efficiency was expressed by the φ_{Eo} fluorescence parameter, which increased to ~110% (*Nicotiana*) or 115% (*Arabidopsis*) of the control at the two highest CM dilutions. Similar stimulation of photosynthesis was previously reported in tests of the autoinduction activity of green alga *Desmodesmus* (earlier *Scenedesmus*) *subspicatus* (Grabski and Tukaj, 2010). However, we observed no significant differences in the φ_{Po} fluorescence parameter (average 0.845) between the control and conditioned cultures of *Arabidopsis* and *Nicotiana*. The probability that an absorbed photon will move an electron beyond Q_A – the primary quinone electron acceptor in PS II (φ_{Eo}), is directly related to the maximum quantum yield of primary photochemistry (φ_{Po}) and the efficiency with which a trapped exciton can move an electron further than Q_A along the electron transport chain (ψ_0) (Strasser et al., 2000). This suggests that the observed increase in φ_{Eo} may be due to improved efficiency of the electron transport chain rather than the quantum yield of primary photochemistry φ_{Eo}. Furthermore, the increase of RC/CS_0 values in the CM/2 and CM/1.25 treatments to ~118% of the control in both tested plants (Fig. 5) indicates an increase in the fraction of active reaction centers in the dark-adapted samples in conditioned cultures. These results are consistent with the observed increase in the photosynthetic pigment content under the influence of conditioned medium (Fig. 4). Our ongoing work aimed at identifying the active factor of CM may yield a better insight into its effect on the photosynthetic apparatus and, in consequence, on plant growth.

CONCLUSION

There is a need for better low-cost methods of improving the growth and productivity of higher plants. Freshwater green microalgae can be maintained on mineral media, and the production of the conditioned (spent) medium (CM) from such cultures is relatively cheap, easy and less time-consuming. Depending on the concentration, algal CM was shown to be a valuable additive improving the growth of tobacco and *Arabidopsis* whole plants cultured in vitro. Our analysis of chlorophyll fluorescence in vivo suggests that the improvement of these plants was due to stimulation of photosynthesis. Our presented and previously obtained results indicate that algal CM offers a very useful protocol for in vitro culture of many commercial varieties, model plants or even wild endangered species.

AUTHORS' CONTRIBUTIONS

EZ designed the research project, prepared the figures and wrote the article; KM-M performed and analyzed fluorescence measurements; KG cultured the algae and prepared the CM; AH and AK performed in vitro cultures of the plants; ZT analyzed and discussed the data.

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