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COMPARATIVE ANALYSIS OF THE RESPONSE OF THREE BULGARIAN VARIETIES OF SPRAY CARNATION TO DROUGHT STRESS, INDUCED BY POLYETHYLENE GLYCOL (PEG-6000)

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Abstract: The response of the in vitro cultures to induced stress enables selection of plants in early stage that are tolerant to water deficit.

The model plants were three Bulgarian cultivars of spray carnation (D*ianthus caryophyllus* f. spray, Hort.:'Biliana', 'Ira' and 'Rusalka'). Polyethylene glycol (PEG-6000) in different concentrations (10%, 20%, 30% and 40%) and for different time durations (1, 3 and 6 days) was used to simulate water deficit in *in vitro* conditions. The response to drought stress was studied using plant growth reactions, relative water content (RWC %), and electrolyte leakage (conductivity). Results showed that the growth of explants in all three cultivars decreased proportionally to the increasing PEG concentrations. For instance, at 30% and 40% PEG the growth decrease was approximately 50% compared to the control. The simulated drought at different concentrations of PEG (10%, 20%, 30% and 40%) induced changes in cell membranes of studied plants. Higher levels of electrolyte leakage on the first day were detected in 'Ira' cultivar (2633±521 µS/g fresh weight at 40% PEG). As a difference in the other 2 cultivars, the highest value was measured at the 6th day of 40% PEG stress ('Biliana' 1864±389.2 µS/g fresh weight and 'Rusalka' – 1712, 8±363 µS/g fresh weight). Relative water content in plant tissues was reduced according to the effect of PEG concentrations. Lowest values of RWC% were recorded at 40% PEG, as in 'Ira' cultivar it was measured on the first day of the experiment (28.52±5,2%), and for 'Biliana' and 'Rusalka' – on the 6th day (respectively 36,4±6,9% and 25,1±2,1%). After the different stress periods spray carnation explants

were placed on MS medium for recovery. The results showed that the plant recovery was 100% after 1 day of drought stress and rooting was observed for all variants of PEG concentrations. On the 6th day, plant explants showed good adaptive response (average of 40%) at high level PEG concentrations (30% and 40%), but explants' rooting was detected only in cultivar Rusalka.

INTRODUCTION

The climate change often causes environmental stress on plants, which reduces the growth and their development, yield and production quality (Marcheva *et al.,* 2013; Spunar *et. al.,* 2008). This opens new challenges for breeders in plant selection programs to study the source material. In that relation it is necessary to apply different methods for determination of resistant cultivars (Schumann *et al.,* 2008; Purohit *et al.,* 1998) in different directions: dry and salt resistance, resistance to diseases and pests, etc.

Drought is one of all unfavorable environmental factors, affecting quality and productivity in growing agricultural crops. The response of plant organisms to the harmful effects of stress factors (including drought) has not been fully elucidated yet, because of plants using a wide range of responses to adapt to changes. Each studied genotype reacts differently to water stress, and even within one species there is a significant change in tolerance to drought (Cicevan *et al.,* 2016).

Studies of physiological mechanisms of plant resistance in laboratory conditions allows the tracking of their specific response to the effect of a single factor. Induced water deficiency by polyethylene glycol 6000 (PEG-6000), which is osmotic with a high molecular weight, allows the plant to dehydrate over a wide range of drought, bringing it as close as possible to the effect of real soil drought (Murillo-Amador *et al.,* 2002). The use of PEG in liquid media allows precision and reproducibility to achieve the desired osmotic potential of the medium (Song *et al.,* 2013).

Studies on ornamental plants were conducted, related to a wider physiological response and their adaptive response to a simulated water deficiency in *in vivo* and *in vitro* conditions (Omami and Hammes, 2006; Yuxiu *et al.,* 2007; Jaleel *et al.,* 2009; Asrar and Elhindi, 2011; Asrar *et al.,* 2012; Zapryanova and Nencheva, 2013; Zapryanova, 2015; Cicevanet *et al.,* 2016).

The aim of the present study was to establish and compare the reaction to drought of three Bulgarian varieties of spray carnation – Biliana, 'Ira' and 'Rusalka in laboratory conditions with the use of different concentrations of Polyethylene glycol 6000.

MATERIALS AND METHODS

For the experiment purpose, the plant material of *D. caryophyllus* f. spray, Hort. cv 'Biliana', 'Ira' and 'Rusalka' was propagated in vitro on MS growth media with added sucrose – 30 g/l and agar 6 g/l at pH = 5.7-5.8 prior to autoclaving (Murashige and Skoog, 1962). Then it was grown in a growth room at a temperature of 22°C, photoperiod of 16:8 (day: night) hours and light intensity 30 $mol.m^{-2}.s^{-1}$.

For the experiment execution, MS medium containing salts and vitamins, sucrose – 30 g/l and polyethylene glycol (PEG 6000) in the following concentrations – 10%, 20%, 30% and 40% with pH = 5.7 was used.

The explants were placed in test-tubes on control (0) and stress inducing liquid medium (PEG – 10%, 20%, 30% and 40%) on filter paper bridges, 10 for each concentration and the control in 3 replications. The duration of stress impact was short (one day), medium (three days) and long-term (six days). Used explants were 2-3 cm high and had a weight of 100-300 mg, measured in sterile conditions prior to their placing in the stress inducing medium. In order to establish the effect of water deficit on plant tissues, we measured the explants' growth and rooting, the rate of cell membranes damage and the relative water content. In *in vitro* study, growth was expressed as the percentage of micro explants' weight increasing after being cultivated for a certain period of time (1, 3 and 6 days) on 10%, 20%, 30% and 40% PEG, compared to the initial weight.

The membrane damage rate was defined by the electrolyte leakage from leaves with accounting for conductivity after stress and was expressed as μS/g fresh weight.

The relative water content (RWC) was measured simultaneously with electrolyte leakage and calculated by the formula of Turner's method (Turner 1981):

RWC% = (fresh weight – dry weight) / (turgor weight – dry weight) X 100

The water deficit (WD) was expressed by the formula:

WD% = 1 – RWC.

After the stress period, explants were transferred to an MS medium without a stress agent (PEG) in order to establish their capacity for recovery and observe the rooting percentage.

Data shown on figures below was expressed as an average value ± SE of two independent experiments, carried out in 10 replications per variant. They were analyzed for significance by means of the t-test of the GraphPad Prizm software. Results showed statistically significant differences at $P<0.05$ (*), $P<0.01$ (**), $P<0.0001$ (***), respectively, as compared to the control.

RESULTS AND DISCUSSION

Applying of short-term osmotic stress (one day) resulted in change in growth indicators, relative water content and cell membrane integrity (**Fig.1**).

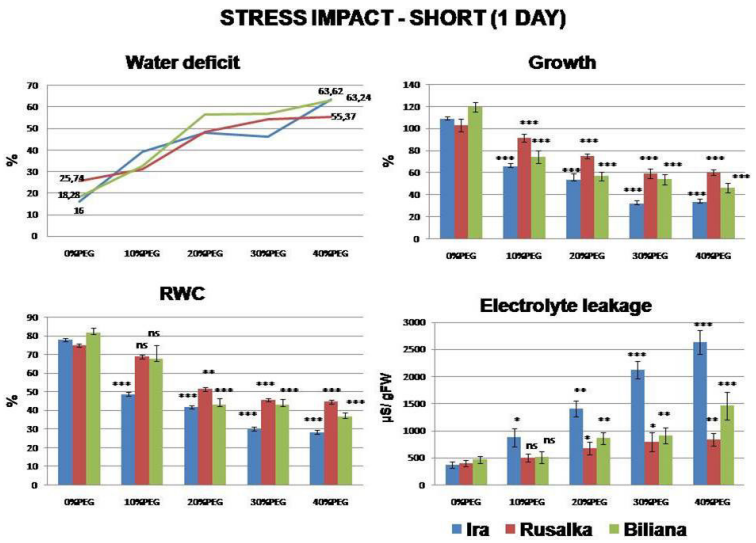

Fig. 1 Physiological indicators of spray-carnation, cv. IRA, RUSALKA and BILIANA in vitro explants at different PEG concentrations and stress duration of 1 day

According to Jalal-Ud-Din and Ali (2009), these physiological parameters can be used as criteria for tolerance to drought in classifying studied varieties. The detected water deficit in control plants varied – 16.36% for 'Ira' variety, 18.28% for 'Biliana' variety and the highest value of 25.74% for 'Rusalka' (**Fig.1**). With the increase of PEG concentration, a specific varietal response was observed. In the cases of 'Ira' and 'Biliana' varieties, there was a sharp increase in the percentage water deficit reported. For example, at 10% concentration of PEG – 39.22% and 32.58%, respectively, while in 'Rusalka' there was a smooth rising. The highest values were reported in variant 40% PEG in all three varieties, for 'Ira' – 63.62%, followed by 'Biliana' – 63.24% and 'Rusalka' – 55.37% (**Fig.1**).

According to the concept of Cornic and Fresneau (2002), dehydration causing plant deficiency up to 30% is considered as mild or moderate stress. Research showed that in our experimental setting plants of the three spray carnation varieties were stressed, which was moderate in low to strong at high concentrations of PEG. This reflected on the reporting of the plant physiological indicators.

As a comparison water deficit on *Tagetes patula* var. 'Usmivka' was studied in two experiment variants: 1th in pots in greenhouse – with reduced watering regime and 2nd in test tubes in laboratory conditions, using different

PEG concentrations. Maximum water deficit (72%) in plant tissues was reported in once a week watering of the pots and in the use of 40% PEG with duration of 6-day stress period. This corresponded to the highest index of electrolyte leakage and RWC in plant tissues, and also reduced plant growth (Zapryanova, 2015; Zapryanova *et al.* 2015).

Explants, grown in the control environment increased their size slightly to 109.1 ± 2.1% for 'Ira', 119.7 ± 4.3% for 'Biliana' and 102.9 ± 5.8% for 'Rusalka'. With the increase of the PEG concentration in the growth medium, growth decreased proportionally in all tested varieties with statistical significance of P <0.0001 *** (**Fig.1**).

Examining the varieties separately, it was found that in 'Ira' variety the growth sharply decreased at concentrations of 30% and 40% PEG, and the reduction was over 70% comparing to the control. In 'Rusalka' variety there was a smooth decrease even at the highest PEG concentration and the difference with the control was about 40%. The 'Biliana' variety was characterized by intermediate values, and comparing to the control, the growth rate was lower with 65% and 73%, respectively (**Fig.1**).

Plants, grown with PEG after the short-term osmotic stress (one day) showed reduced turgor, but retained their green color (**Fig.1**). Growth inhibition was associated with the reduced ability of plants to absorb water (Shabani *et al.,* 2013). Osmotic stress at the cellular level slows cell division (Levitt, 1980). The cells lose their turgor and this leads to weight loss (Heyser and Nabors, 1981).

In 'Zhoro' *Chrysanthemum* variety with PEG-induced water deficiency, wilting occurred at low concentrations of 10% and 20% PEG. At high concentrations of 30% and 40% PEG growth inhibition and differences with the control with over 50% were established (Zapryanova and Nencheva, 2013).

The same tendency was found in *Tagetes* 'Usmivka' variety. With the increase of PEG concentration in growth medium, the growth of the explants decreased proportionally. At concentration of 30% and 40% PEG, the growth was below 50% compared to the control 54.88 ± 2.5 μS/g fresh weight – at 30% and 57.16 ± 6.5 μS/g fresh weight at 40% PEG with statistical significance P <0.0001 (Zapryanova, 2015).

The control plants of three spray carnation varieties showed a high percentage (about 80%) of water content in their tissues. The results obtained showed that the applied PEG stress lead to a gradual reduction of water content in plants (Fig.1).

In 'Ira' variety, the measured RWC values sharply decreased even at low percentage of PEG. At 10% PEG they decreased by 29% compared to the control. The minimum value was reached at 40% PEG – 28.62%, which was almost half of the value, measured in untreated plants (Zapryanova *et al.,* 2016).

'Biliana' variety responded with a 15% decrease of RWC at low PEG concentration (10%) compared to the control. At 20% and 30% PEG values were retained, and at 40% PEG they reached 36.76%, which was 45% lower than the control.

The ‘Rusalka’ variety was characterized with a gradual decrease in water content in plant tissues without drastic sudden changes (Ivanova *et al.,* 2018). The difference in the values between the control and those of 10% PEG-treated plants was 6%. The minimum value was detected at 40% PEG – 44.62%, which was the highest value of all three tested varieties.

Detection of electrolyte leakage in the control plants and in three varieties in the short-term stress (one day) was in low values (approximately 400 µS/g fresh weight) (**Fig.1**). This corresponded to the normally expected reaction of the tested material. The simulated drought in the experiment showed an increase in electrolyte leakage at all PEG concentrations used. Only in ‘Ira’ variety there was a sharp rising in this indicator at low PEG content (10%). The highest values were reported in the one-day stress of 40% PEG in ‘Ira’ variety – 2633 ± 521 µS/g fresh weight (Zapryanova *et al.,* 2016), and the lowest in ‘Rusalka’ variety 800 µS/g fresh weight (Ivanova e*t al.* 2018). The ‘Biliana’ variety was characterized by a smooth increase in this value. At 40% PEG it reached 1460 µS/g fresh weight, which was 30% higher than the control (**Fig.1**). In this regard, a number of studies indicate that cell membranes are the initial sites of stress effect (Munns *et al.,* 2006).

In the three-day stress period, changes occurred in the individual test parameters (growth, water content in plant tissues and cell membrane integrity) that gave an idea for the reaction of each tested variety of spray carnation (Fig.2). Unlike the short-term stress period, the changes in all three varieties pass smoothly without sharp decrease or increase (**Fig.2**). At 40% PEG the maximum water deficit was measured, which varied from 59% to 75%. This showed that plants were under heavy stress. In contrast to the short-term stress period, in which ‘Rusalka’ variety was characterized by the lowest value of the water deficit – 55.37%, three-day stress period showed the highest value of 75%. In the other two varieties – ‘Ira’ and ‘Biliana’, a slight decrease of 60% and 58% occurred (**Fig.2**)

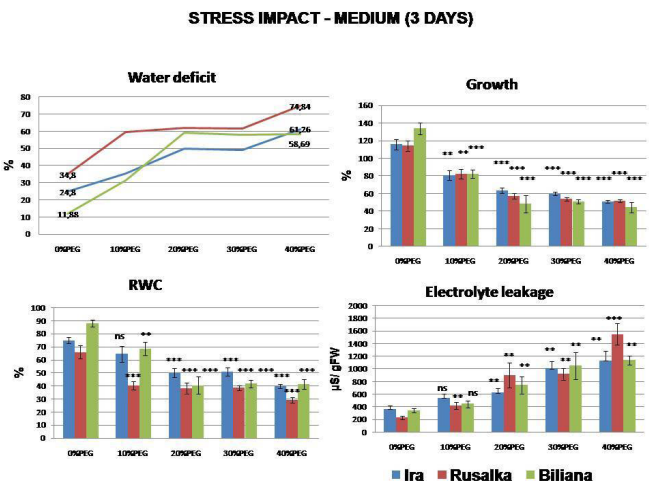

Fig. 2 Physiological indicators of spray-carnation, cv. IRA, RUSALKA and BILIANA in vitro explants at different PEG concentrations and stress duration of 3 days

Growth inhibition was found in all three varieties. The lowest value was recorded for 'Rusalka' – 51%, compared to the control where the decrease was 63% (Ivanova et al., 2018). The control plants of 'Biliana' variety showed the highest growing rate of 133.8%, while in 40% PEG, the growth was three times lower. In 'Ira' variety, the decreasing was 43% compared to the control (**Fig.2**).

The relative water content in plant tissues also decreased. The lowest values were recorded for 'Rusalka' variety – 29.25%, and the highest for 'Biliana' variety – 41.31% (**Fig.2**). Thus compared to the short-term stress period, where 'Ira' variety was characterized by the lowest value of the RWC – 28,327%, during the three-day stress period, it showed an increased value – 39.74% (Zapryanova *et al.,* 2016). Changes in the RWC were probably due to structural and functional changes, ensuring the adaptation of plants to drought.

In studies on drought resistance of *Amaranthus* plants it was found that in control plants the RWC kept a high rate of tissue water content. For instance the RWC of A. tricolor was between 83% and 92% and 86-94% in *Amaranthus cruentus*. Stress had caused a significant change in the reported values, and this indicator in *Amaranthus tricolor* decreased up to 58% and for *A. cruentus* up to 60% (Omami and Hammes, 2006).

In our experiments on spray carnation, electrolyte leakage rates at different PEG concentrations remained high at 3 day stress, compared to those of the control plants. However, at 10% and 20% PEG concentrations recorded during the one-day stress in all three tested varieties the electrolyte leakage rate was lower (Fig. 2).

Increase of values were noted at high PEG concentrations of 30% and 40%, as the highest values were recorded in 'Rusalka' variety – 1553.3 μS/g fresh weight and the lowest – in 'Ira' variety 1128 μS/g fresh weight. In 'Biliana' variety, a decrease in electrolyte leakage rate was found to be 1142 μS/g fresh weight compared to one-day stress period, which value was 1460 μS/g fresh weight. The increased water content in tissues and the reduced electrolyte leakage found in the 'Ira' and 'Biliana' varieties were indicators of growing adaptation of plants to the induced water deficit (**Fig.2**).

The results of water deficit on the sixth day exceeded three to four times the values of the control plants (**Fig.3**). For 'Ira' variety at 30% PEG it was registered 69% and 71% at 40% PEG. For 'Biliana' variety values were 53.2% at 30% PEG and 63.59% at 40% PEG, and in 'Rusalka' variety they were 74.84% at 30% PEG and 76.33% at 40% PEG (**Fig.3**).

Progressive water deficit caused a decline in the growth of all three varieties of spray carnation. With prolongation of the treatment period (6 days), the reported growth values decreased compared to the control. At 40% PEG for 'Ira' variety it was 43.47 ± 2.8%, for 'Biliana' – 43.43 ± 5.9% and for 'Rusalka' – 30.52 ± 2.7% with statistical significance at $P < 0.0001$ (**Fig.3**).

The control plants on the sixth day were fresh, green and in a normal turgor condition. In plants, grown at 10% and 20% PEG concentration a slight withering was observed, whereas in 30% PEG and 40% PEG the withering increased significantly. Necrotisation of 10% of explants of 'Biliana' variety was observed, while the same was not noticed in 'Ira' and 'Rusalka' varieties.

'Biliana' plants were found to have the highest RWC % in the 6-day stress period – 36.41%. The lowest value of RWC% was recorded for 'Rusalka' – 25.16% (**Fig.3**).

A similar response was observed in *Carthamus tinctorius* L. (saffron) callus culture, the lowest growth rates and RWC% were reported with the use of 40% PEG (Kakaei *et al.,* 2013). The relative water content in plant tissues of Tagetes 'Usmivka' variety and 'Zhoro' *Chrysanthemum* also decreased and the lowest values were reached on 6th day at 40% PEG – 27.26 ± 6.4% and 28.52 ± 5,2%, respectively (Zapryanova and Nencheva, 2013; Zapryanova, 2015)

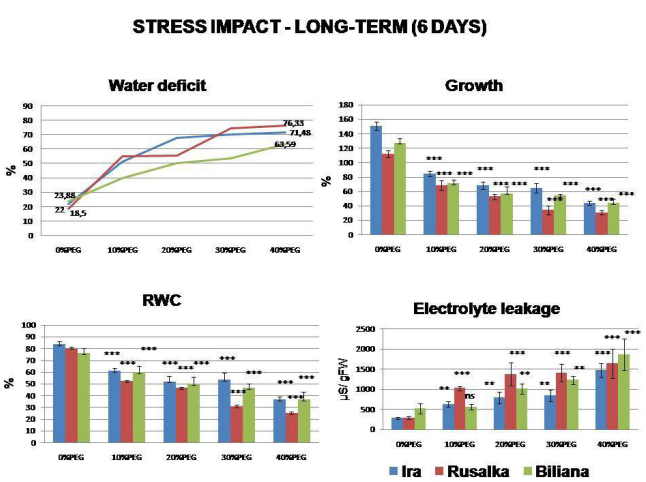

Fig. 3 Physiological indicators of spray-carnation, cv. IRA, RUSALKA and BILIANA in vitro explants at different PEG concentrations and stress duration 6 days

In 'Ira' variety, there was also a decrease up to 36.38% observed (Zapryanova *et al.,* 2016). However, compared with the one-day stress period, there was an increase in this indicator. That showed that 'Ira' variety responded sharply to the applied stress at the initial stage and then gradually adaptation to the osmotic stress conditions. In the other varieties – 'Biliana' and 'Rusalka', the reaction to the water deficit was intensifying during its extended period of action (**Fig.3**).

RWC is a key indicator of the degree of cell and tissue hydration, which is essential for optimal physiological functioning and growth processes (Silva *et al.,* 2007). In drought stress, the cell membrane undergoes changes affecting the permeability and its stability (Blokina *et al.,* 2003). According to Vendruscolo *et*

al. (2007), resistant varieties keep higher RWC than expected, so they probably maintain hydration of the protoplasts for a longer period of time under dry conditions, thus providing better adaptability (Sikuku *et al.,* 2012).

In 'Zhoro' *Chrysanthemum* variety, the prolonged stress of drought lead to decrease in turgor, stable wilting of plants and tissue necrosis, especially at higher PEG concentration. In control plants values were constant – about 70%, but at 40% PEG on the 6th day they reached 18.10 ± 3.6% (Zapryanova and Nencheva, 2013).

A slight difference was observed between 'Biliana' and 'Rusalka' varieties at the cell membrane integrity values of 1864 μS/g fresh weight and 1712 μS/g fresh weight respectively. In 'Ira' variety, simulated drought also caused leakage of electrolytes – 1471 μS/g fresh weight, but this was much lower level compared to the obtained during the one-day stressful period (**Fig.3**).

Simulated drought, using different concentrations of polyethylene glycol 6000 (10%, 20%, 30% and 40%), induced changes in *Tagetes* cell membranes. Highest electrolyte leakage rates were recorded at 6th day 4625 ± 521 μS/g fresh weight at 40% PEG (Zapryanova, 2015).

After various stress periods of PEG concentration, plant explants of spray carnation were placed to restore on MS medium. The results for 'Ira' variety showed that in the short stress period (1 day) at different PEG concentrations, the recovery and rooting was 100%. For medium-term stress (3 days), 100% recovery was obtained at concentrations of PEG – 10%, 20% and 30%, while at 40% concentration only 80% of the plants were restored. Rooting was best at 10% PEG, reaching 80%, while at higher concentrations (30% and 40% PEG) it was only 10% rooting. Long-term (6-day) PEG stress showed 100% recovery at concentrations of 10% and 20% PEG, but the amount of rooted plants was reduced up to 50%. At high concentrations of PEG – 30% and 40% the recovery was 60% and 40%, respectively, and rooting was not registered (Zapryanova *et al.,* 2016).

The results obtained for 'Rusalka' variety showed that in short stress period (1 day) the recovery was 100%. Rooting at low PEG values was 100%, whereas at high PEG concentrations the percentage of rooted plants decreased up to 75% for 30% PEG and 60% for 40% PEG (Ivanova *et al.,* 2018).

For medium-term stress (3 days), 100% recovery was obtained at concentrations of 10%, 20% and 30% PEG, while at 40% PEG – 90% of the plants were recovered. Rooting was best demonstrated at 10% PEG, which was 90%, while at higher concentrations – 30% and 40% PEG it was 50% and 30%, respectively. Long-term (6-day) PEG stress showed 100% recovery at concentrations of 10%, 20% and 30% PEG, while the quantity of rooted plants decreased up to 50%. For 40% PEG the recovery was 30% and rooting was recorded in only 10% of the explants.

'Biliana' variety was recovered 100% after short stress period (1 day) at different PEG concentrations. Rooting was recorded in all variants, regardless of the PEG concentration. At 40% PEG rooted plants were 90%. For medium-term stress (3 days) at 10%, 20% and 30% PEG – 100% recovery was observed, while at 40% PEG the recovery was reduced to 60%. Best rooting – 70% was recorded at 10% PEG, while at 40% PEG there was only 10% rooting. Long-term (6-day) PEG stress showed 100% recovery at concentrations of 10% and 20% PEG. However, the quantity of rooted plants decreased up to 50%. At the high concentrations of PEG – 30% and 40% the recovery was 50% and 30%, respectively, but rooting was not recorded.

Tagetes plant explants showed a good adaptive response to a short-term treatment. High recovery rate was recorded at 30% and 40% PEG treatment – 60% and 40%, respectively. The long-term stress period of 6 days unfavorable affected the Tagetes explants. Only 10% recovered plants were obtained at 40% PEG and no rooting was recorded (Zapryanova, 2015).

CONCLUSIONS

Spray carnation varieties 'Ira', 'Biliana' and 'Rusalka' reacted individually on the simulated drought stress, using PEG (10%, 20%, 30% and 40%) in controlled (*in vitro*) conditions – as the detected water deficit varied between 30% to 70%. Changes in explant growth, cell membrane stability and the RWC in plant tissues were established, corresponding to the stress duration and the PEG concentrations. The usage of 40% PEG, used for 6 days was the maximum admissible for spray carnation, which was based on the low percentage of explant rooting (10%), reported only for 'Rusalka' variety.

AUTHORSHIP STATEMENT

NZ - designed the experiment, performed the experiments /collected the data, analyzed the data, wrote the manuscript. MY - analyzed the data, wrote the manuscript. II -performed the experiments/collected the data, analyzed the data. BA - designed the experiment

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