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IMPROVEMENT OF THE POLLEN QUALITY AND GERMINATION LEVELS IN GRAPES (*VITIS VINIFERA* L.) BY LEAF PULVERIZATIONS WITH NANOSIZE CALCITE AND SEAWEED EXTRACT (*ASCOPHYLLIUM NODOSUM*)

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ABSTRACT

Studies indicated that toxic materials such as pesticides, plant bio-stimulants and chemical fertilizers used in intensified agriculture are detrimental to the viability of the plant pollen and cause reduction in plant fertility. Pollen quantity, viability and germination capability are important parameters for both fruitfulness and breeding studies. Therefore, effects of 100% organic materials, namely nanosize calcite ($CaCO_3$, SiO_2, MgO, and Fe_2O_3) and seaweed extract (*Ascophyllium nodosum*) pulverizations on size, fertility and germination rates of the pollens of 'Thompson Seedless' and 'Narince' grapevine cultivars were investigated. The calcite treatment, alone or along with seaweed improved viable pollen (dark red) percentages that varied from 65.(control) to 77.9% (combined treatment) in 'Thompson Seedless', and from 55.3% (control) to 68.8% (calcite) in 'Narince'. The improvement of pollen germination due to calcite was more pronounced in pollens of 'Narince' than in 'Thompson Seedless'. In general, slight changes in lengths of polar and equatorial axises of grape pollens in response to the treatments were also observed. The correlation between pollen viability and germination percentages was different among the cultivars. Overall, leaf pulverizations of nanosize calcite and seaweed extract had remarkably positive effects on grapevine pollen characteristics and therefore both applications could be recommended as environmental-friendly practices in sustainable viticulture.

Key words: Pollen quality, sustainable practices, leaf fertilizers, grapevine.

INTRODUCTION

The need to feed an ever-increasing population is a constant pressure on crop production in the world. Intensification in production is generally associated with the increased use of external inputs. In such intensified production techniques, many may have had some undesirable effects on environments and water quality (Kara and Sabir, 2010). Global warming is becoming a major part of the political agenda. Studies have indicated that toxic materials as well as climate change detrimentally influence the morphological structure of the plant pollen that reduce pollen fertility and subsequent yield (Speranza *et al*., 2013), since the pollination and subsequent fertilization are essential issues for fruit set in plants, proper development of floral organs and adequate pollen production with high viability and germination capability are the primary requirements for fertilization (Kelen and Demirtas, 2003; Naveed *et al*., 2014) for both fruitfulness and breeding studies (Sabir, 2010). While genetic factors determine the ultimate potential of the pollinizer (Hill *et al*., 1985), environmental variables, including mineral nutrition of the plant, have profound influence the quantity and quality of the pollen produced and its subsequent performance (Nyomora *et al*., 2000; Hussein *et al*., 2014). The plants are exposed to multiple abiotic stresses rather than one particular stress simultaneously in field conditions. The combination of high temperature and water stress represents an excellent example of multiple abiotic stress occurring concomitantly in the field (Wu *et al*., 2012). In the world, a large proportion of vineyards are located in regions with a risk of drought (e.g. Mediterranean-type climates) and high temperature, that constitute serious constraints on grape yield and quality. It is also believed that the frequency of extreme events such as heat waves or global warming is predicted to increase (Kuster *et al*., 2013). In many other crop species, the effects of high temperature stress are more prominent on reproductive development than on vegetative growth and the sudden decline in yield with temperature is mainly associated with pollen infertility (Zinn *et al*., 2010).

Research work has mainly focused on the adverse effects of biotic stress on pollen quality of various plants (Naveed *et al*., 2014). However, little information is available regarding the effects of organic cultural practices, such as leaf nutrient pulverization, on pollen quality of horticultural plants. For a sustainable and environmentally healthy agriculture, researchers have tested the effectiveness of many organic materials such as seaweed extracts (Khan *et al*., 2009), nanosize produces (Kara and Sabir, 2010), and agent microorganisms (Sabir *et al*., 2012a; Chen *et al*., 2013; Sabir, 2013) on plant growth. Marine algal seaweed species have often been regarded as an underutilized bioresources (Khan *et al*., 2009). Among the most commonly used seaweeds, *Ascophyllum nodosum* L. is the most researched and used for nutrient supplements and as bio-stimulants to increase plant growth (Sabir *et al*., 2014). The seaweed *A. nodosum* contain polysaccarides, laminaran, fucoidan, and hormonal substances (Lane *et al*., 2006), all of which have been reported to have a wide range of biological activities (Rioux *et al*., 2007) such as growth stimulation, natural defense, and antimicrobial properties (Fritig *et al*., 1998). Similarly, a 100% natural product containing nano size particles of Ca, Si, Mg and Fe have also been reported to have growth promotion effects in various aspects (Kara and Sabir, 2010; Sabir *et al*., 2012b). Promoting the vegetative development via leaf pulverization of such natural products supplemented with nutrients and/or plant hormones would presumably assure robust development of floral organs and provide better yield under water scarcity. Therefore, this study was conducted to determine the influence of nanosize calcite and seaweed extract (*A. nodosum*) on pollen characteristics (polar axis length and equatorial axis length), fertility and germination rates of grapevine cultivars 'Narince' and 'Thompson Seedless'.

MATERIALS AND METHODS

Growth condition and treatments: In this study, eight years old healthy and productive vines of 'Narince' (an important and widespread wine variety in Turkey) and 'Thompson Seedless' (the most important raisin cultivar in the world, known as international synonyms such as 'Sultana', 'Sultanina' or 'Sultani Cekirdeksiz') were evaluated in relation to single or combined treatments of nano size calcite product [$CaCO_3$ (40%), SiO_2 (4%), MgO (1%), and Fe_2O_3 (1%)], and seaweed extract (*A. nodosum*) for pollen characteristics. The leaves of the first group vine were pulverized with nanosize calcite (0.5 g L^{-1}) while the second received seaweed extract (0.3 g L^{-1}). Besides, a third group was treated with mixture of both the components, while the fourth control group received neither of the treatments. Each experimental group consisted of 12 vines in equal growth. The application concentration of each product was prepared according to the company instructions. The first treatment was performed when the shoots were around 10 cm and a total of three pulverizations per treatment were carried out with 7 days intervals. The vine are planted in east-west oriented rows with the spaces 2 x 3 m. Cultivation practices were performed as commonly practiced by the local growers. In canopy management, shoot positioning was done according to the unique growth habits of each cultivar in vertical shoot positioned trellis system. The experimental vines received the same cultural practices such as weed control, and pruning and the vineyard was rain-fed with a limited precipitation around 50±5 kg m^{-2} occurred up to the bloom (Anonymous, 2011).

Pollen sampling and measurements: Before the beginning of flowering, the inflorescences (clusters) were isolated with bags. The pollen samples were obtained by cutting the flowers at the beginning of the blooming period (Marasali *et al*., 2005) and brushing the anthers and pollen into an eppendorf tube using a soft brush (Gallardo *et al*., 2009). The investigations on pollen characteristics like lengths of polar and equatorial axises were carried out using 60 fresh pollen grains for each treatment group (Chkhartishvili *et al*., 2006).

The viability level of pollen was determined using 900±60 pollen grains (in nine different area) for each treatment group. This level was determined using 1% of TTC (2,3,5-triphenyl tetrazolium chloride) test (Norton, 1966). The solution prepared with this chemical, which is normally colorless, turned into triphenylformazan, which is insoluble and appears red in living tissue to which it is applied. This reaction is formed by some reductase enzymes in living tissue. The activity of redox-enzyme occurs via the same mechanism in other tetrazolium salts, such 2,3,5 triphenyl tetrazolium chloride. Therefore, according to the level of liveliness in tissue, tissues stain red in accordance to the density of enzyme activity. Hence, a dark color is observed with increased enzyme activity as compared to a the light color observed with decreased enzyme activity (Smith, 1951). One drop of this solution was placed on a slide, pollen grains were spread with a brush on the slide, and a cover slip was placed on top. After TTC application, eight randomly chosen area (8 replications) on each microscope slide were used for counting the pollens. Pollen grains that stained dark red were referred to as viable, light red as semi-viable, and yellowish green plus unstained as non-viable (Eti, 1991).

In vitro pollen germination capacity was determined with saturated petri method (Eti, 1991; Kelen and Demirtas, 2003). The fresh pollen harvested from experimental vines were hydrated and incubated at 20 °C in petri plates on a medium containing 15% sucrose, 1% agar supplemented with or boric acid at 100 mg L^{-1}, and adjusted to pH 5.8. Pollen germination was recorded after 6 h of incubation as the percentage of germinated pollen in a total of 100±15 grains from different areas of the petri plate. Pollen was considered to have germinated if the pollen tube was twice as long as the diameter of the pollen grain (Nyomora *et al*., 2000). For each treatment, two petri dishes and six regions in each petri were investigated (Bayazit *et al*., 2011) using a fluorescence microscopy (Euromex microscopes Holland), and percentages of germination were determined.

Data evaluation and statistical analysis: Analysis of variance was performed to determine the effects of the treatment on the different parameters. The treatments mean were compared using the Tukey significant difference test. Differences were considered significant at

$p<0.05$. All the statistical analyses were performed using the SPSS-17 software package (SPSS Inc., Chicago, IL, United States). Pearson's correlation analysis was performed to detect the relationships between pollen viability level and germination percentages.

RESULTS AND DISCUSSION

Pollen viability: All the treatments significantly increased the percentages of dark red (viable) pollen (Table 1) in 'Narince' cultivar with the highest dark red pollen (68.8%) in vines treated with nano size calcite, followed 65.3% by vines treated with seaweed extract. By contrast, the light red (semiviable) and unstained (non-viable) pollen decreased in response to the treatments. The pollen viability response of 'Thompson Seedless' was quite different (Table 2) with the highest viability (77.9%) with combined application, followed by seaweed (73.4%), while the least value was observed in control (65.5%). The control groups for both the cultivars had the highest percent values for unstained pollens. The pollen viability of various fruit species may exhibit significant variation even under similar conditions (Eti, 1991). In a study with eight grapevine cultivars, Kelen and Demirtas (2003) observed 31.5 and 68.8% pollen vaibility. In this study, the treatments had profound effects on pollen viability despite the genotype-dependent differences. As known, Fe and Mg are the structural components of many enzyme systems in plants and in their nano size can provide higher stability and efficiency to the enzyme systems (Millaleo *et al*., 2010). Therefore, nano size Fe and Mg particles in calcite treatments had positive effect on photosynthesis physiology (Sabir *et al*., 2012b). Improved photosynthesis may provides higher soluble sugars in the anther walls and ultimate increase in pollen viability (Sabir *et al*., 2015).

Pollen germination: All the treatments significantly increased the pollens germination percentages of both the cultivars, except the seaweed extract in 'Thompson Seedless', (Fig. 1, 2). In 'Narince' cultivar, the germination ranged from 49.6% for pollen of vines treated with calcite to 40.3% for those of untreated vines (control). As for 'Thompson Seedless', the combined application of calcite and seaweed resulted in the highest germination percentage (25.6%), followed by calcite alone (24.6%). The pollen germination percentage may vary greatly in grapevine cultivars. For example, 'Burgund' and 'Muscat Ottonel' had the highest germinated pollen percentages of 62.0 and 71.72%, respectively while, the germintaion percentages were as low as 21.74 and 28.09% for 'Muscat Hamburg' and 'Coarnă neagră' on saturated Petri (Blidariu and Dobrei, 2012). Using the same in vitro conditions, Similarly, Tangolar *et al*. (1999) reported wide variability in pollen germination, with 11.4% in 'Thompson Seedless and 39.1% in 'King's Ruby' grapevine cultivars. The germination percentages observed on 'Narince' fell is within the ranged observed earlier for different cultivars, while the pollens of 'Thompson Seedless' seem similar to those cultivars having low germination percentages as indicated by Tangolar *et al*. (1999).

Pollen size: The pollen morphology has become an important descriptor since the advent of the scanning electron microscope (Perveen and Qaiser, 2003), as the pollen has its own unique set of characteristics such as size, exine structure, and pore size or number (Evrenosoglu and Misirli, 2009). The polar and equatorial axis lengths of pollens of the two cultivars were significantly affected by the treatments (Table 3, 4). Nano size calcite treatment resulted in the highest polar axis length (28.6 and 28.4 µ for 'Narince' and 'Thompson Seedless', respectively). Similar positive effect of calcite on equatorial axis was also found for both cultivars. Seaweed extract had slight influences on pollen sizes. On the other hand, combined application of the two materials had no positive effect in general, except for enhanced polar axis of 'Thompson Seedless' pollens. The pollen sizes investigated in this study are in conformity of the findings of previous studies. For example, Chkhartishvili *et al*. (2006) studied the peculiarities of pollen grains in eight autochthonous grapevine genotypes and the polar axis length varied from 28.4 to 35.0 µm, and the length of equatorial axis from 14.7 to 17.2 µm. Apart from genetic characteristics, the development of angiosperm pollen depends completely on the sporophyte for the provisioning of nutrients. It is, therefore, reasonable to expect that growth conditions of the sporophyte may influence the quality of pollen. Lau and Stephenson (1993) revealed a profound effect of nutrients on the size of pollen grains produced by two zucchini cultivars (*Cucurbita pepo*). In one of the previous studies, Brewbaker *et al*. (1963) revealed the essential role of calcium ion among the nutrients, and its action confirmed in 86 species representing 39 plant families. They further indicated that the high requirement of calcium and low calcium content of most pollen may conspire to give calcium a governing role in the growth of pollen tubes both in vitro and in situ. The role of Ca, as an essential nutrient, required for pollen tube growth has been appreciated for many years. Experiments using $^{45}Ca^{2}$ demonstrated that Ca is taken up by pollen (Jaffe *et al*., 1975; Bednarska, 1989), and inhibition of Ca uptake results in the rapid arrest of pollen tube growth. It is also well established that Ca plays a key role in the regulation of pollen tube growth (Feijo *et al*., 1995).

Correlations between viability and germination: In pollens of 'Narince' cultivar, a positive correlation ($R^2=0.647$, $p<0.01$) was found between the viable (dark red stained) pollen and germination percentages (Fig. 2). However, there was a significant negative correlation

(R^2=-0.564, p<0.01) between semi-viable (light red) pollen and germination (Fig 3). Therefore, higher pollen germination percentages was only due to the increase in red light stained pollens. Such cases were quite different in 'Thompson Seedless'. The correlation between pollen viability and germination percentages in 'Thompson Seedless' were 0.068 for dark red pollen and germination, and 0.023 for light red and germination. Such extend values imply that the cultivars responded differently to the treatments in relation to pollen quality characteristics.

Although a linear relationship is expected between viability and germination ability, there have been certain contradictory reports. For example, Nybom (1985) reported that the germination level was not significantly correlated with pollen viability in some blackberry species while Beyhan and Serdar (2008) revealed a linear relationship between these parameters in pollens of chestnut. Hence, the results of the present and illustrated studies reveal a genotype-specific aptitude in relation to germination power of viable pollens.

Table 1. Changes in pollen viability of 'Narince' in response to the treatments.

Treatment	Pollen viability level percentages (%)		
	Dark red (%)	Light red (%)	Unstained (%)
Control	55.3±0.51 c	22.4±0.56 a	22.4±0.31 a
Calcite	68.8±0.75 a	16.7±0.35 c	14.5±0.50 d
Seaweed	65.3±1.02 b	17.3±0.67 c	17.5±0.31 b
Combined	64.3±0.55 b	19.8±0.83 b	15.9±0.35 c

Within column, means followed by a different letter differ significantly at $p < 0.05$ by Tukey.

Table 2. Changes in pollen viability of 'Thompson Seedless' in response to the treatments.

Treatment	Pollen viability level percentages (%)		
	Dark red (%)	Light red (%)	Unstained (%)
Control	65.5±0.51 c	20.0±0.40 b	14.4±0.15 a
Calcite	66.2±1.47 c	25.8±0.95 a	8.0±0.28 b
Seaweed	73.4±1.57 b	20.1±1.10 b	6.5±0.33 c
Combined	77.9±1.47 a	13.5±0.13 c	8.6±0.74 b

Within column, means followed by a different letter differ significantly at $p < 0.05$ by Tukey.

Table 3. Changes in lengths of polar and equatorial axises of 'Narince' in response to the treatments. Means followed by a different letter differ significantly at $p < 0.05$ by Tukey.

Treatment	Polar axis (μ)	Equatorial axis (μ)
Control	26.9±0.38 b	16.3±0.46 bc
Calcite	28.6±0.09 a	17.9±0.37 a
Seaweed	26.7±0.34 b	16.9±0.42 b
Combined	23.9±0.19 c	15.6±0.09 c

Within column, means followed by a different letter differ significantly at $p < 0.05$ by Tukey.

Table 4. Changes in lengths of polar and equatorial axises of 'Thompson Seedless' in response to the treatments. Means followed by a different letter differ significantly at $p < 0.05$ by Tukey.

Treatment	Polar axis (μ)	Equatorial d axis (μ)
Control	27.1± 0.33 ab	15.3±0.29 c
Calcite	28.4±0.58 a	16.7±0.05 a
Seaweed	26.8±0.44 b	15.6±0.10 bc
Combined	28.1±0.21 a	15.8±0.17 b

Within column, means followed by a different letter differ significantly at $p < 0.05$ by Tukey.

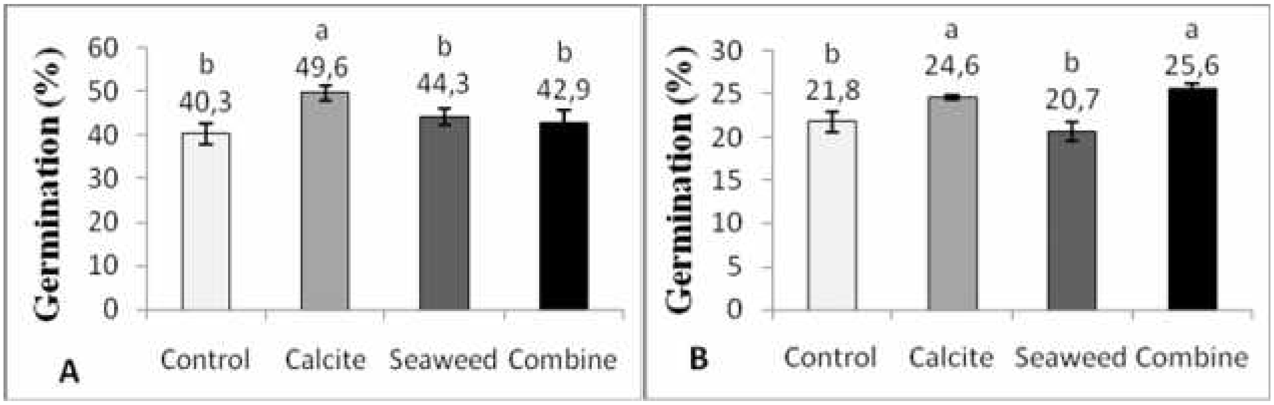

Fig. 1. Changes in pollen germination percentages (%) of 'Narince' (A) and 'Thompson Seedless' (B) in response to the treatments. Means followed by a different letter differ significantly at *p* < 0.05 by Tukey. Error bar stands for the standard deviation of that mean.

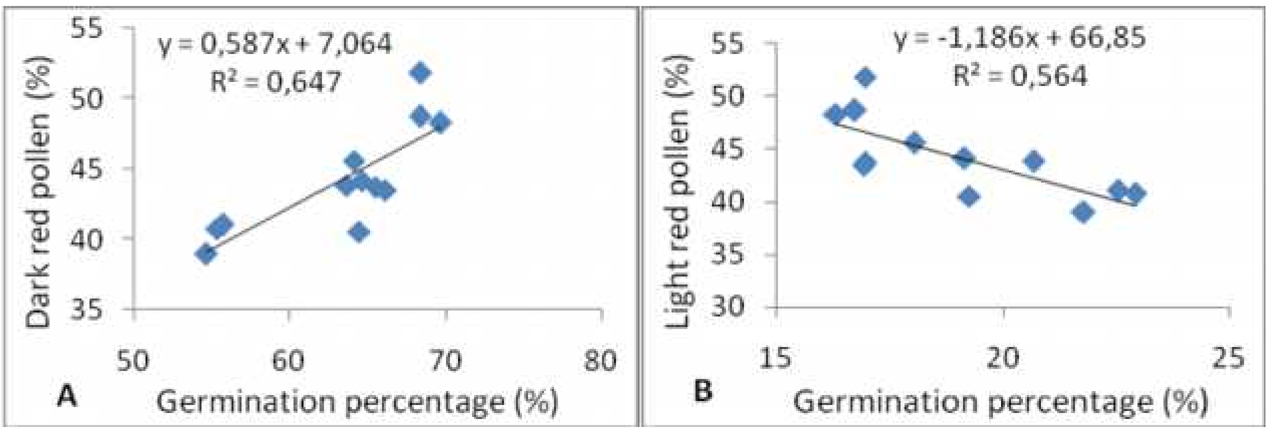

Fig. 2. Correlation of germination percentages with dark red (A) and light red (B) stained pollen in 'Narince'.

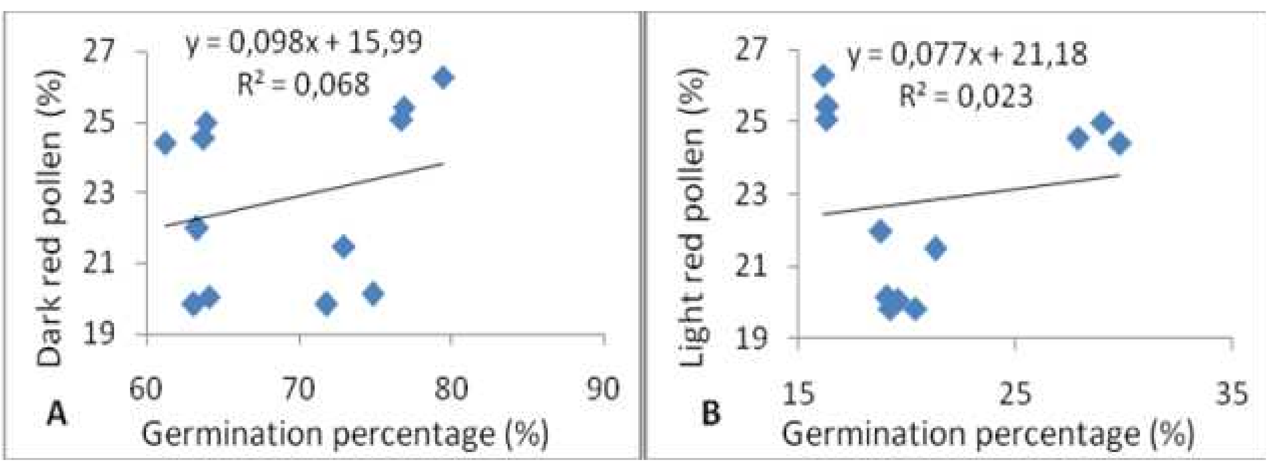

Fig. 3. Correlation of germination percentages with dark red (A) and light red (B) stained pollen in 'Thompson Seedless'.

Conclusion: According to the literature investigations on pollen, previous studies have mainly focused on the negative effects of biotic stress on pollen quality of various plants. However, there is insufficient knowledge in literature on the improvement of pollen quality in plants despite the fact that abiotic stresses and climate change may adversely affect the pollen health and may decline the subsequent yield. In this study, leaf pulverizations of nanosize calcite and seaweed extract had remarkable positive effects on grapevine pollen characteristics and therefore both two applications could be recommended as environmental-friendly practices in sustainable crop productivity. It can also be deduced from the general results that the cultivars responded differently to the treatments although the treatments were beneficial in most cases. The application of eco-friendly practices in sustainable agriculture would also alleviate the restraints of biotic and abiotic stress factors on the plants.

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