



Usage of Nano-Particle NPK to Reduce the Amount of Mineral Fertilizers in 'Crimson Seedless' Grapevines

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Abstract

Fertilization through the addition of nano-fertilizers, added in very small quantities as a foliar spray on the vine canopy, has become one of the most effective methods in order to reduce the amount of mineral fertilizers. Therefore, this experiment was conducted on a 7 year-old "Crimson seedless" grapevines for successive seasons 2021 and 2022 to determine the optimum rates of "Nano-NPK" fertilizer to replace the "NPK" conventional fertilizer. Three concentrations of "Nano-NPK" were used as foliar application in three concentrations, Nano - NPK (200, 30, 200 ppm), Nano-NPK (400, 60, 400 ppm) and Nano-NPK (800,120, 800 ppm) replacing 50% of conventional fertilizer NPK compared to the soil addition of the conventional fertilizer NPK at (25, 25, 100 kg/Feddan) as a control. Our results showed that Nano-NPK fertilizer at (800, 120, 800 ppm) induced a significant increase in yield parameters, along with the chemical and physical characteristics of the vegetative growth as compared with the lower concentrations but there are no significant differences between it and the control. Thus, application of Nano-fertilizers was very effective in accelerating the clusters growth stages along with replacing the mineral fertilizers

Keywords: Nano-NPK, Nano-fertilizer, Crimson seedless, Grapevine.

Introduction

Table grape cultivar “Crimson seedless” (*Vitis vinifera L.*) is a late red cultivar with excellent fruit qualities, good natural flavor, as well as strong and crispy berries. It has a great market acceptance due to its excellent nutritional properties and its exportable value (Río-Segade *et al.*, 2013). Therefore, it is critically important to adopt sustainable farming practices that optimize the use of chemical fertilization for crop nutrient requirements to minimize the risk of environmental pollution by testing other methods of fertilization using new technologies such as nanotechnology (Manjunatha *et al.*, 2016).

Nano-fertilizers (NFs) due to their very low size of particles have higher surface area which are smaller than the leaves pores. They are synthesized or modified form of the mineral fertilizers bulk materials or extracted from different vegetative or reproductive parts of the plant by different chemical, physical, mechanical or biological methods using nanotechnology (Singh *et al.*, 2017). Numerous studies have shown that nanotechnology has had a positive effect in increasing production of agricultural crops, reducing excessive fertilizer use and clearing the soil of heavy elements. Furthermore, they are less costly when applying nano fertilizer in agricultural as an effective supplement to conventional fertilizers in smaller amounts, in addition to their long periods of storability due to their high stability under different conditions (Ali and Al-Jodhry, 2017). Foliar spraying of NPs improves the effectiveness of plant protection technologies compared to traditional soil-root application as they mainly enter through the leaf stomata, and transported to different plant parts via apoplastic and symplastic pathways (Hong *et al.*, 2021)

In general, nano-fertilizers increase penetration and uptake of nutrients (Nair *et al.*, 2010) and dry matter content (Ditta and Arshad, 2015). As well, NFs are known to prevent plants from different biotic and abiotic stress where it builds carbon uptake (Manjunatha *et al.*, 2016), plants are able to keep their stomata closed longer when they are subjected to water stress, result better crop yield and food quality and enhance soil fertility (Qureshi *et al.*, 2018). Nitrogen (N) is one of the major nutrients that plants need in large quantities and the main forms of nitrogen

absorbed by plants are often in the form of nitrate (NO_3^-) or ammonium (NH_4^+). Generally, the nitrate is immediately absorbed but is easily leached from the soil. Therefore, it is preferable to add nitrogen in the form of ammonia, as the plant takes it slower and then is converted into nitrate through nitrification. When nitrogen is added in excess or insufficient quantities it can cause negative effects on plant productivity. One of the most important symptoms of its deficiency is the appearance of pale green leaves that tend to yellow (Grapevine Nutrition Literature Review, 2022)

Nano-fertilizers combine nano devices in order to synchronize the release of Fertilizer-N and -P with their uptake by crops, so preventing undesirable nutrient losses to soil, water and air via direct internalization by crops, and avoiding the interaction of nutrients with soil, microorganisms, water, and air (De Rosa *et al.*, 2010). Previous studies reported that foliar application of NPK Nano-fertilizer induced marked significant increases in all growth variables determined at fully vegetative and reproductive growth stages (Abdel-Aziz *et al.*, 2016).

Likewise, Phosphorus is one of the most important elements and a key for life for plant growth as its role is critical since it is associated with photosynthesis, cell formation, enlargement and cell division and involving in the formation of cellular membranes (Malhotra *et al.*, 2018). Potassium is of relevant interest as it influences grapevine growth, berry composition, as well as must quality. However, K^+ content of grape berries increased steadily over the last decades, in part due to climate change as the properties and qualities of many fruits are also impacted by environment.

Potassium nutrition is of great importance as it impacts the grape growth, berry chemical and physical composition and quality. However, the steadily increasing in K^+ content of grape berries over the past decades is due to climate change which affected the properties of many fruits (Villette *et al.*, 2020).

Obviously, deficiencies in major minerals like potassium (K^+), nitrogen (N), and phosphorus (P) strongly affect metabolism with subsequent impacts on plant growth, crop yield, nutritional value, and composition of grape berries

(Amtmann and Armengaud, 2009; Fernandes *et al.*, 2016).

This experiment was aimed to assess the beneficial effect of using foliar NPK nano-fertilizer in different rates as an alternative to reduce the conventional NPK fertilizer doses through replacing them partly and its impact on the vegetative growth and productivity in 'Crimson seedless' grape cultivar.

Materials and methods

This assessment took place during two successive seasons 2021 and 2022 on 7-year-old 'Crimson seedless' (*Vitis vinifera* L.) grapevine in a commercial vineyard located at "Sadat city", Menoufia governorate, Egypt at 30° 22' 30" N and 30° 30' 1" E and altitude of 247 m. Vines are spaced at 2 × 3 m., trellised by Spanish parron system and grown in a sandy soil and irrigated via a drip irrigation system. A completely randomized block design was used, with sixty vines uniform in vigor (4 treatments x 3 replicates x 5 vines/replicate). Vines were cane pruned on the 1st week of February, with a bud load of 100 buds/vine (10 canes x 10 buds/cane) in addition to 5 spurs x 2 buds / spur. The experiment was carried out on the same vines for both seasons 2021 and 2022, and received common horticultural practices recommended by Ministry of Agriculture.

The conventional NPK preparation

Ammonium nitrate (33.5 % N), mono calcium superphosphate (15.5 % P₂O₅) and potassium sulphate (50 % K₂O) were used as source of N, P and K respectively with the recommended dose of NPK mineral fertilizer at a rate of (25, 25, 100 kg / Feddan, soil addition) as a control. This amount was reduced to 50% with the foliar application of the nano-NPK treatments.

NPK nano-fertilizer preparation and characterization

Chitosan (CS) (molecular weight 50,000-190,000 Da, degree of deacetylation 75-85% and viscosity: 20-300 cP), acetic acid, sodium triphosphate (TPP), Tween 80, phosphoric acid (85%), potassium sulphate and urea. All chemicals used in this study were purchased from Sigma-Aldrich, USA chemical company and used without further purification.

Chitosan-NPK nano-fertilizer (CS-NPK NF) was prepared according to Corradini *et al.* (2010) with some modifications. Briefly, CS aqueous solution

(0.2% w/v) was prepared by dissolving CS in acetic acid solution (1% v/v) at room temperature. The pH of the solutions was adjusted to 5.5 with 0.5 M NaOH solution. Subsequently, TPP solution (0.02% w/v) was added dropwise to CS solution under vigorous stirring for 30 min. NPK nano-fertilizer was prepared by loading nitrogen (N), phosphorous (P) and potassium (K) into chitosan nanoparticles by dissolving different amounts of NPK into 100 ml of nanoparticle solution under homogenizing at 18000 rpm for 30 min in presence of Tween 80 at 25°C. The resulting solution to incorporate NPK into the nanoparticles presents this final concentration: i) 200, 400 and 800 ppm of N; ii) 30, 60 and 120 ppm of P; iii) 200, 400 and 800 ppm of K. Dynamic light scattering (DLS) measurement of size and Zeta Potential was undertaken using a Nano-zetasizer (Malvern, ZS Nano, UK). The morphology of CS-NPK NF was imaged by High Resolution Transmission Electron Microscope (HR-TEM) operating at an accelerating voltage of 200 kV (Tecnai G2, FEI, Netherlands). Diluted CS-NPK NF solution was ultra-sonicated for 5 min to reduce the particles aggregation. Using micropipette, three drops from the sonicated solution were deposited on carbon coated-copper grid and left to dry at room temperature. HR-TEM images of the Chitosan-Urea nanocomposite that deposited on the grid were captures for morphological evaluation. Synthesis and characterization of Chitosan-Urea nanocomposite was performed in Nanotechnology & Advanced Materials Central Laboratory (NAMCL), Agricultural Research Center (ARC), Giza, Egypt.

Each rate was divided and added whether as foliar spray or soil addition four time along the growing season as follow: the first 10% N, 65% P, 30% K after bud burst, the second 0% N, 15% P, 10% K at blooming, the third 75% N, 15% P, 55% K after berry set till veraison and the fourth 15% N, 5% P, 5% K after harvest.

The fertilization treatments involved:

1. NPK mineral fertilizer (25, 25, 100 kg/Feddan) as a control.
2. Nano - NPK (200, 30, 200 ppm)
3. Nano-NPK (400, 60, 400 ppm)
4. Nano-NPK (800, 120, 800 ppm)

After harvest, when total soluble solids reached about 16 -18%, according to Tourky et al. (1995), the clusters were taken to Laboratory and samples of 15 clusters were collected randomly from each treatment, (5 clusters from each replicate) and the following measurements were taken to evaluate the effect of the different treatments:

1. Yield and clusters characteristics

- Yield per vine (kg): to calculate the average yield/vine, the random samples were collected and weighed then the mean of cluster weight was multiplied by the number of clusters / vine.
- Average cluster weight (g)
- Average berry weight (g)
- Average berry size (cm³)

2. Chemical characteristics of berries

- Refractometric total soluble solids (TSS %) in berry juice was determined by using a hand refractometer.
- Titrateable acidity % (one gram of tartaric acid for 100 ml of juice) according to (AOAC, 2000)
- TSS / acid ratio
- Anthocyanin content in berry skin (mg/100g F.W) was estimated according to (Yildiz and Dikem, 1990)

3. Physical characteristics of the vegetative growth

- Leaf area (cm²): Samples of 20 leaves randomly collected from the mature basal leaves of the sixth and seventh nodes from each treatment for leaf area determination at harvest time (using leaf area meter, Model CI 203, U.S.A.).
- Shoot length (cm): it was determined by measuring the fruiting shoots before harvest.
- Pruning weight in (kg): at winter pruning

4. Chemical characteristics of the vegetative growth

- Total chlorophyll content of leaves (SPAD): were measured at harvest time in the mature

basal leaves of the sixth and seventh nodes by using portable Minolta chlorophyll meter model SPAD 502.

- Percentage of N, P and K content in leaf petioles were estimated in the leaf opposite to the cluster, N (%) content were measured according to (Hesse, 1971), P % was measured referring to the method of Schouwenburg and Walinga (1967) and K % was determined using a flame photometer (Jackson, 1973)
- Total carbohydrates in canes (%) were estimated according to Smith *et al.*, (1956)

5. Statistical analysis

The statistical analysis of the present data was carried out according to Snedecor and Cochran (1980). Averages were compared using the new L.S.D. values at 5% level.

Results and discussion

Dynamic Light Scattering (DLS) Analysis

DLS was used to measure hydrodynamic diameter in the nanometer range. The size of CS-NPK NF was 28 nm and zeta potential 44.5 mV (Figure 1).

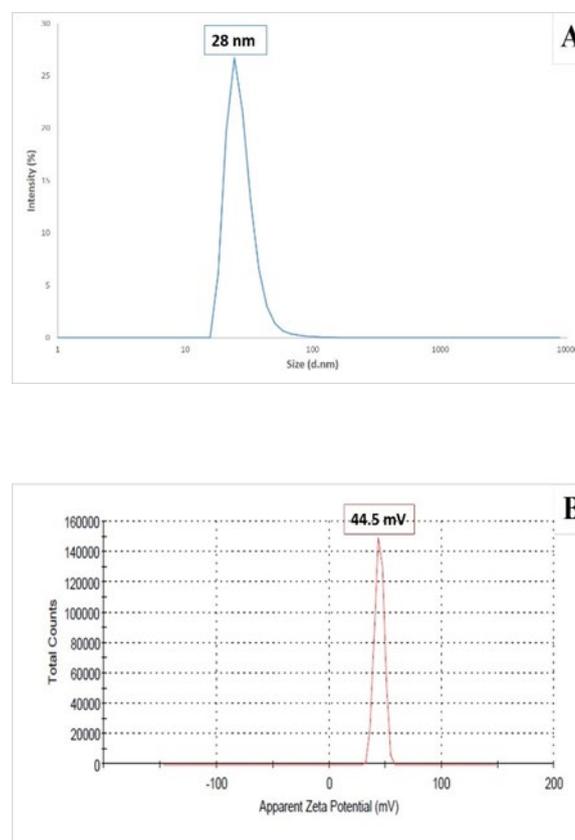


Figure (1). DLS analysis of CS-NPK NF. Particle size (A), and Zeta potential (B).

Transmission electron microscope (TEM) analysis result

Transmission electron microscope (TEM) gave us information on the particle shape and the determination of particle size. Typical TEM micrograph of the CS-NPK NF was shown in Figure 2. CS-NPK NF has nearly spherical shape, smooth surface and average size about 26.6 nm.

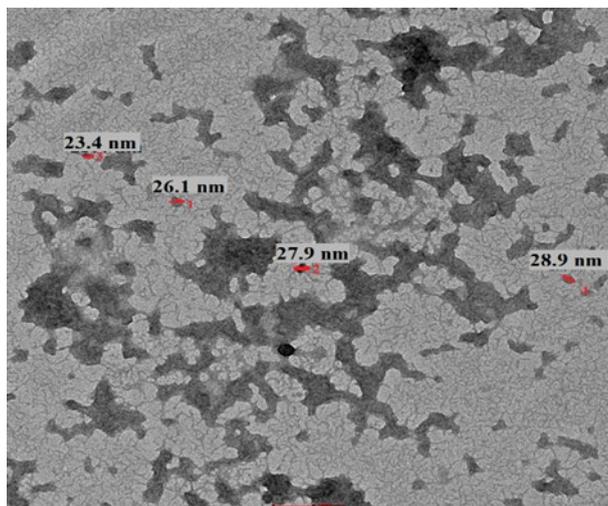


Figure (2). TEM image of CS-NPK NF.

The effect of Nano-Particle NPK on different measurements

1. Yield and clusters characteristics

Average yield/vine (Kg), average cluster weight (g), average berry weight (g), and average berry size (cm³)

Clearly data in table (1) shows the average yield per vine as affected by all treatments. Generally, increases in yield tend to result from increases in cluster weight due to increasing the berry weight in both seasons in all treatments. It is obvious that in the case of treating the vines with Nano-NPK at (800, 120, 800 ppm) resulted in larger berries producing the highest yield followed by the lower concentration (400, 60, 400 ppm) then the control

respectively with a slightly significant differences between the lowest concentration (200, 30, 200 ppm) and the control in term of average yield/vine, cluster weight, berry weight and berry size in both seasons. This detection is being linearly related to those of Thakur *et al.* (2008) reported that in "Perlette" grapevines, the foliar application of potassium increased berry weight. Likewise, Tanou *et al.*, (2017) who mentioned that Nano-fertilizers increase the diffusion and dissolution of nutrients and thus their availability to the plant, which leads to an increase in photosynthesis besides the Nitrogen effects in increasing the efficiency of the plant to carry out the process of photosynthesis in conjunction with the element phosphorus and stimulating the production of Auxin, which encourages cell division and elongation of cells, which tend to produce large berries increasing the yield per vine. This increase in weight may be attributed to higher cell division and photosynthetic activities (Kumaran *et al.*, 2019).

2. Chemical characteristics of berries

Total soluble solids (TSS %), titratable acidity (TA %), TSS / acid ratio and total Anthocyanin

Influence of N, P and K treatments upon berries chemical characteristics is given in table (2). Total soluble solids content of juice was significantly influenced by all the fertilizers. The maximum TSS % content was recorded with Nano-NPK at (800, 120, 800 ppm) treatment. Application of P and K fertilizers resulted in a progressive increase in TSS content with augment in dose of these fertilizers. However, the highest potassium level increased the TSS content of the juice. Overall, a significant effect was noted due to either of nitrogen, phosphorus and potassium rates on acid content. This is consistent with the work of Ibrahim *et al.* (2019), who reported that the foliar spraying treatments with Nano elements gave a significant effect on

Table (1). Effect of different rates of NPK nano particles fertilization and the conventional form on Yield and its attributes of Crimson seedless grapevine during the two successive seasons 2021 and 2022.

Treatments	Average yield (Kg)		Cluster weight (g)		berry weight (g)		Berry size (cm ³)	
	Season 2021	Season 2022	Season 2021	Season 2022	Season 2021	Season 2022	Season 2021	Season 2022
Conventional NPK (control) (25, 25, 100 kg/Fed)	16.9	17.5	508.5	522.3	4.75	4.89	4.53	4.62
Nano - NPK at (200, 30, 200 ppm)	15.9	16.1	489.0	497.4	4.33	4.38	4.15	4.20
Nano-NPK at (400, 60, 400 ppm)	18.7	20.0	554.3	572.1	5.20	5.27	4.97	5.06
Nano-NPK at (800, 120, 800 ppm)	20.0	21.7	572.4	601.5	5.49	5.55	5.37	5.44
New L.S.D at 5%	1.0	1.1	20.0	21.0	0.16	0.18	0.22	0.28

TSS, TSS/acid ratio of Superior seedless grapevines. Previous reports indicate that an increase in level of Nano-K concentrations increased significantly all chemical characteristics of berries in term of TSS, TSS/acid ratio and total anthocyanin of 'Flame seedless' grapevines (Bedrech and Farroh, 2022). Likewise, increasing the amount of K fertilization caused a significant increase in total soluble solids and TSS/acid ratio and a decrease of acid concentration, they suggested that increasing K fertilization improves sugar transport into the berries Bedrech *et al.* (2022).

Additionally, some studies revealed that foliar applications of potassium increased anthocyanin content on 'Crimson Seedless' (Mohsen, 2011). In another trial, Topalović *et al.* (2011) stated that foliar sprays with a liquid fertilizer containing potassium and phosphorus increased the total anthocyanin content of 'Cardinal' grape cv.

3. Physical characteristics of the vegetative growth

Leaf area (cm²), Shoot length (cm), Pruning weight in (kg)

It is obvious from the data displayed in (table, 3) that there are significant differences among treatments in the physical characteristics of the vegetative growth parameters in term of leaf area, shoot length and the weight pruning. The highest values were obtained from treating the vines by the higher concentration of the NPK-nano (800,

120, 800 ppm) followed by (400, 60, 400 ppm) and the conventional NPK at (25, 25, 100) with no significant differences between them. It was found that the reason for the increase in the leaf area can be due to the increase in the enzymatic activities and their reactions as a result of the use of nano-fertilizers, which leads to the production of raw materials that increase cell divisions and then increase the leaf area (Hatami *et al.*, 2014). It is also noticeable that there was a significant effect for the mentioned treatment on shoot length, as the values were higher than the other treatments as stated by Doaa *et al.*, (2019) who declared that treating the vine by a combination of 75% conventional potassium and 250 ppm Nano-K gave a significant increase in shoot length over using conventional k alone. In this respect, Doaa and Raesa, (2020) scrutinized the impact of N-nano at uncovering that the leaf area was greater at the higher concentration (1000 ppm) than both lower concentrations (500 and 250 ppm) in 'Ruby seedless' grapevine.

Likewise, Faker and Obaid, (2020) showed that there were significant differences in spraying nano-NPK fertilizer on grapevine leaves, recorded the highest leaf area while the control at (0 mg L⁻¹) recorded the minimum. Aside from increasing the leaf area and shoot length, NPK-nano gave the Highest Pruning weight when applied on Citrus (Al-Jilhawani *et al.*, 2020). Also, it was revealed that the application of K nano increased the shoot fresh weight of Marigold flower, (Erfani *et al.*, 2021).

4. Chemical characteristics of the vegetative growth

Table (2). Effect of different rates of NPK nano particles fertilization and the conventional form on the chemical characteristics of berries of Flame seedless grapevine during the two successive seasons 2021 and 2022.

Treatments	TSS (%)		TA (%)		TSS / acid Ratio		Anthocyanin Mg/100 g. FW	
	Season 2021	Season 2022	Season 2021	Season 2022	Season 2021	Season 2022	Season 2021	Season 2022
Conventional NPK (control) (25, 25, 100 kg/Fed)	15.4	15.7	0.60	0.58	25.6	27.0	29.2	29.4
Nano - NPK at (200, 30, 200 ppm)	14.8	15.0	0.66	0.62	22.4	24.1	25.5	25.7
Nano-NPK at (400, 60, 400 ppm)	16.5	16.8	0.54	0.53	30.5	31.6	31.1	33.5
Nano-NPK at (800, 120, 800 ppm)	17.2	17.9	0.43	0.42	40.0	42.6	38.9	39.8
New L.S.D at 5%	0.5	0.6	0.04	0.03	2.6	2.2	3.1	3.7

a. Total chlorophyll content in leaves (SPAD)

The chlorophyll content in leaves is an important factor that can be affected by nitrogen application. As a result, we found significant differences between all treatments receiving N according to the degree of concentration whereby the higher the nitrogen concentration the higher the chlorophyll content (table, 3). The application of NPK-nano at (800, 120, 800 ppm) showed an increase in leaf chlorophyll content over all other treatments and as a result of the N applications. Keller *et al.*, (2001b) also recorded an increase in leaf chlorophyll content as a result of the N applications on Pinot Gris Grapevines. The nitrogen is mostly related to green pigment of leaves thus chlorophyll was used as nitrogen status indicator (Muñoz-Huerta *et al.*, 2013).

b. Percentage of N, P and K content in leaf petioles (%)

The leaf petiole N content, as estimated through the chlorophyll content of the leaves (SPAD), was measured during the two growing seasons of the study and SPAD values were presented in Table (4). The concerned results elucidate that the higher and lower values of N, P and K % ranged between (N 2.60, P 0.20, K 1.53 and N 2.85, P 0.30, K 1.75) for the first season and (N 2.61, P 0.21, K 1.58 and N 2.89, P 0.36, K 1.76) for the second season respectively with a superiority for the higher concentration of NPK-nano (800, 120, 800 ppm) signaling the important of increasing Nitrogen amount in rising N % in leaf petioles. It is also apparent that all

Nano K fertilizer treatments significantly increased N and K leaf petioles compared to control treatment in both seasons of study. Similar to this finding Al-Moshileh and Al-Rayes (2004) stated that a significant increase in leaf petiole content of N and K was observed as potassium fertilizer rate was increased. Likewise, Doaa and Raeesa, (2020) investigated the effect of applying N-nano foliar fertilizer at 1000 ppm along with 50% soil addition of conventional N on 'Ruby seedless' grapevine finding its significant impact on N, P and K concentration in leaf petioles, compared to control treatment.

c. Total carbohydrates in canes %

Obtained values revealed that there were significant differences among treatment in both seasons (table, 4). The highest total carbohydrates percentage in canes was recorded by applying NPK-nano (800, 120, 800 ppm) followed by NPK-nano (400, 60, 400 ppm), these result was attributed to vines receiving high concentration of K which can travel easily throughout the vine and involve in carbohydrate transport and metabolism. Meanwhile, application of NPK fertilization had significant effects on carbohydrate content of canes as the highest rate of N, P or K fertilization increased carbohydrate content from (17.9 to 21.1 %) and (18.4 to 21.5 %) compared to the control in both seasons of the study respectively. Carbohydrate accumulation correlated with photosynthesis efficiency and leaf chlorophylls since high chlorophyll results in high content of Carbohydrate (Köse, 2014). Results similar were obtained from Aly *et al.* (2020) who studied the

Table (3). Effect of different rates of NPK nano particles fertilization and the conventional form on the physical characteristics of the vegetative growth of Crimson seedless grapevine during the two successive seasons 2021 and 2022.

Treatments	Leaf area (cm ²)		Shoot length (cm)		Pruning weight (kg)	
	Season 2021	Season 2022	Season 2021	Season 2022	Season 2021	Season 2022
	Conventional NPK (control) (25, 25, 100 kg/Fed)	157.1	162.9	145.9	148.7	2.83
Nano - NPK at (200, 30, 200 ppm)	149.8	157.6	143.7	146.9	2.75	2.89
Nano-NPK at (400, 60, 400 ppm)	178.2	181.8	151.2	155.6	2.99	3.19
Nano-NPK at (800, 120, 800 ppm)	195.1	197.8	155.4	160.8	3.12	3.25
New L.S.D at 5%	13.0	14.0	3.0	3.1	0.11	0.12

Table (4). Effect of different rates of NPK nano particles fertilization and the conventional form on the chemical characteristics of the vegetative growth of Crimson seedless grapevine during the two successive seasons 2021 and 2022.

Treatments	NPK	Total chlorophyll (SPAD)		N %	Leaf petiole content				Total carbohydrates %	
		Season 2021	Season 2022		Season 2021	Season 2022	Season 2021	Season 2022	Season 2021	Season 2022
		Conventional (control) (25, 25, 100 kg/Fed)	28.5		29.8	2.65	2.69	0.22	0.26	1.58
Nano - NPK at (200, 30, 200 ppm)	26.2	27.0	2.60	2.61	0.20	0.21	1.53	1.58	17.4	18.0
Nano-NPK at (400, 60, 400 ppm)	29.9	31.6	2.77	2.81	0.25	0.30	1.67	1.69	19.5	19.8
Nano-NPK at (800, 120, 800 ppm)	35.4	35.7	2.85	2.89	0.30	0.36	1.75	1.76	20.1	21.5
New L.S.D at 5%	0.8	0.9	0.05	0.06	0.02	0.01	0.05	0.06	0.4	0.5

effect of potassium phosphate on 'Crimson seedless' cv. observing a rise in the values of total carbohydrates % over the control. These results coincided with those of Parveen (2021) who mentioned that Potassium, is a crucial element which intensifies the synthesis of carbohydrates. In addition, Phosphorus plays an important role in carbohydrates translocation (García-Caparrós, 2021).

Conclusion

In conclusion, we can deduce that treating the vines by nano-materials is directly affecting the nutrients rate as well as improving their absorption efficiency. Hence, nanoparticle fertilizers have diameters less than the pore size of the cell wall of plant therefore, NPK-nano have the ability to enter easily through it and reach up

to the plasma membrane. The current study focused on the effect of partly replacing the conventional NPK by NPK-nano in order to reduce the up taken amount besides reducing the costs. Obtained results showed that the higher concentration of foliar spray of NPK-nano (800, 120, 800 ppm) with 50% of the conventional NPK was the most effective treatment in increasing all growth parameters as well as enhancing the chemical characteristics of berries and leaves. Thus, using NPK in the nano form has a beneficial effect in reducing the amount used and consequently the costs of fertilizers.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this study.

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