








Article

Urea-Doped Calcium Phosphate Nanoparticles as Sustainable Nitrogen Nanofertilizers for Viticulture: Implications on Yield and Quality of Pinot Gris Grapevines

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Abstract: In recent years, the application of nanotechnology for the development of new “smart fertilizers” is regarded as one of the most promising solutions for boosting a more sustainable and modern grapevine cultivation. Despite showing interesting potential benefits over conventional fertilization practices, the use of nanofertilizers in viticulture is still underexplored. In this work, we investigated the effectiveness of non-toxic calcium phosphate nanoparticles ($\text{Ca}_3(\text{PO}_4)_2 \cdot n\text{H}_2\text{O}$) doped with urea (U-ACP) as a nitrogen source for grapevine fertilization. Plant tests were performed for two years (2019–2020) on potted adult Pinot gris cv. vines grown under semi-controlled conditions. Four fertilization treatments were compared: N1: commercial granular fertilization (45 kg N ha^{-1}); N2: U-ACP applied in fertigation (36 kg N ha^{-1}); N3: foliar application of U-ACP (36 kg N ha^{-1}); C: control, receiving no N fertilization. Plant nitrogen status (SPAD), yield parameters as well as those of berry quality were analyzed. Results here presented clearly show the capability of vine plants to recognize and use the nitrogen supplied with U-ACP nanoparticles either when applied foliarly or to the soil. Moreover, all of the qualitative parameters measured in vine plants fed with nanoparticles were perfectly comparable to those of plants grown in conventional condition, despite the restrained dosage of nitrogen applied with the nanoparticles. Therefore, these results provide both clear evidence of the efficacy of U-ACP nanoparticles as a nitrogen source and the basis for the development of alternative nitrogen fertilization strategies, optimizing the dosage/benefit ratio and being particularly interesting in a context of a more sustainable and modern viticulture.

Keywords: nanofertilizers; grapevine nutrition; nitrogen; grape quality; grape volatile compounds; sustainable viticulture

1. Introduction

The most recent challenge of viticulture is moving towards an enhanced sustainability, meaning a reduction of the chemical inputs for the vine management and a better use of natural resources [1,2]. Among the agronomic practices, fertilization, plant protection

against pests, and weed control are the main causes of the intensive use of agrochemicals in vineyards [3,4]. A proper managing of fertilizer application in the field can be one of the greatest challenges, since it focuses on maximum nutritional efficiency of fertilizers to enhance crop yield and ensure environmental safety. Excessive levels of nutrients, especially N and P, can be subjected to leaching along soil profile or volatilization, causing, in turn, water pollution and hazardous gaseous emissions [5–7]. It is interesting to note that since 1961 the use of nitrogen fertilizers has increased by 800% [8]. As a consequence, the CO₂ equivalent emissions from synthetic nitrogen fertilizers in EU 28 in the 2018 was 71.203 gigagrams (FAOSTAT 2021) [9].

Considering both the crucial role of fertilizers to ensure an equilibrate yield and quality in vineyards and the need to limit their negative side effects, it appears evident how urgent is to revise the concept of fertilizers and fertilization management, in a vision of feeding vines rather than soil. A range of agronomic practices are pursued to improve the efficiency of fertilizers, by tuning the timing and availability of nutrients to plants or by optimizing the placement and the fertilization rate on the base of the vine nutritional needs [10].

It is interesting to note that the so-called ‘intelligent fertilizers’, releasing promptly and locally the nutrients according to the plants’ requirements, can surely represent a promising route for an improved nutrient use efficiency. Among them, slow- and controlled-release fertilizers comprise coated, water-insoluble or slowly water-soluble products [11,12], while stabilized fertilizers are amended with additives that reduce the transformation rate of the inorganic nutrients, resulting in an extended time of availability in the soil [13]. In most of these fertilizers the slow release is limited to the nitrogen or phosphorus, and the duration of the nutrient release may vary from 3 up to 18 months [14]. Several studies have demonstrated the effectiveness of these fertilizers in terms of nutrient sources in different crops [15–17]. Moreover, the integration of slow-release fertilizers with their localized application (precise fertilization) can surely be a more effective and flexible tool for an appropriate vine nutrition management [18].

In recent years, application of nanotechnology for the development of new types of fertilizers is regarded as one of the most potentially promising options for boosting a more sustainable grapevine production [19,20]. Nanomaterials having size <100 nm are generally highly reactive, due to their small dimensions and their high surface to volume ratio, compared to bulk materials. Moreover, specific properties of nanomaterials (e.g., crystallinity, size, morphology, zeta potential, etc.) contribute to improve their colloidal stability and ionic strength in solution, increasing the bioavailability of nutrients to the microorganism-root system [21]. Thanks to these physical, chemical, and biological properties, the combination of nanomaterials and fertilizers results in products able to guarantee an increased and effective acquisition of nutritional elements by plants [22]. In this respect it is interesting to mention that nanofertilizers, obtained by either nanoparticulated nutrients or nutrients encapsulated/coated with nanomaterials for controlled or slow delivery of macro- and micronutrients, have proven to provide important benefits in the fertilization management of several herbaceous and tree crops [23,24].

In grapevine, Mozafari et al. [25] reported that the *in vitro* application of iron nanoparticles in Khoshnaw grapes optimized iron nutrition while increasing the plant resistance to drought stress. Hamed Wassel et al. [26] investigated the effects of foliar application of six nanofertilizers (amino minerals, Orgland, active—Fe, Boron—10, Amino—Zn, and Super—Fe) to Flame Seedless Grapevines, showing enhanced growth, vine nutritional status, yield and quality for vines when fed with nanofertilizers.

With respect to nitrogen, a crucial element for grapevine production and wine quality, it should be noted that the evidence of the possible use of nanotechnologies in the supply of this nutrient in viticulture is still very limited. Interesting results were reported for herbaceous crops by using amorphous calcium phosphate (ACP) nanoparticles functionalized with urea (U-ACP), the most commonly used N fertilizer [27–29]. As nanoparticles, ACP show a high specific surface area and a higher reactivity than their crystalline counterparts (e.g., nano-apatites). These properties allow loading ACP surface with significant amounts

of N-dopants, like urea and nitrate ions, which are released slower as compared to highly soluble conventional fertilizers; this is the key feature that favors the gradual uptake of N by the plants [28,29]. Additionally, ACP are intrinsically rich in Ca and P. Their high solubility in neutral or slightly acidic media enables a higher delivery of these important ions to the plant [30], providing the opportunity to employ these nanoparticles also as multinutrient nanofertilizers. The use of ACP as fertilizers in agriculture has been proven to be safe as long as bioavailability, movement in soils and human toxicity issues are considered [31,32]. While investigating the potential exposition to different nano-calcium phosphate materials, including (undoped) ACP, Epple et al. [32] concluded that “under all reasonable conditions, calcium phosphate nanoparticles can be considered as safe for humans”.

In a recent study, Pérez-Álvarez [33] tested a foliar application of U-ACP on field-grown grapevines cv Tempranillo. Authors reported that the grapes harvested from plants treated with U-ACP provided quality levels similar to those treated with a conventional foliar fertilizer (urea), despite a considerable reduction of nitrogen dosage. Increased yeast assimilable nitrogen (YAN) and amino acid concentration were found in U-ACP treated grapes, both classes of compounds having significant impact on fermentation kinetics and wine sensory quality.

While showing interesting potential benefits of U-ACP over conventional foliar fertilization practices, the results of the mentioned research work still leave several open questions regarding the application of the U-ACP nanotechnology for grapevine fertilization. In fact, the use of urea-doped nanoparticles has been tested as a complement in the fertilization plan so far, but no studies have investigated the effectiveness of this nanomaterials as a N source to be used as an alternative to conventional fertilizers. Moreover, the effect of these nanofertilizers on yield, berry macrostructure, as well as on grape aromatic profile (which are all largely influenced by nitrogen availability [34–36]), are still unknown.

Considering then the limited information still available on the use of nitrogen nanofertilizers in grapevine, the present work aims at evaluating the capability of urea-doped calcium phosphate nanoparticles (U-ACP) to maintain grape yields and quality at restrained nitrogen dosages. Plant tests were performed for two years in potted adult Pinot Gris cv. vines grown under semi-controlled conditions (ambient temperature/radiation and controlled water supply). Fertigation and foliar application of U-ACP nanoparticles were tested and compared to a conventional granular fertilization and to a non-fertilized control, in order to evaluate the effect of different U-ACP application techniques. Relevant yield parameters (i.e., yield, bunch number, bunch weight) and quality parameters of berries (i.e., sugar content, titratable acidity, fingerprint of volatile compounds) were analyzed at harvest. Overall, this work aims to extend knowledge on the use of calcium phosphate nanoparticles as nanofertilizers, pursuing a novel and more sustainable strategy for nutrition management in vineyards.

2. Materials and Methods

2.1. Production and Characterization of U-ACP Nanofertilizers

The preparation of U-ACP nanofertilizers was carried out according to the protocol reported by Carmona et al. [30]. Technical grade reagents were purchased from on-line distributors: calcium nitrate, potassium nitrate and urea from ALFE Natura (Italy, www.alfenatura.com), dipotassium hydrogen phosphate from MyProtein (UK, www.myprotein.com, accessed on 20 May 2021), sodium citrate dihydrate from Algin-Chemie (Germany, www.algin-chemie.de), and sodium carbonate from buXtrade (Germany, www.buxtrade.de, accessed on 20 May 2021). The purity of the reagents was assessed by X-Ray Powder Diffraction (XRD) [30] prior to use. In a typical preparation, an aqueous solution ($V = 75$ mL) of calcium nitrate (0.4 M) and sodium citrate (0.4 M) [solution A] was poured into an aqueous solution ($V = 75$ mL) containing dipotassium hydrogen phosphate (0.24 M), sodium carbonate (0.2 M) and potassium nitrate (0.4 M) [solution B]. The mixture was heated at 37 °C for 5 min. The resulting suspension was centrifuged (10 min, 4500 rpm) and washed (300 mL \times 2). The slurry obtained from two preparations was mixed with

a solution of Urea (1 g in 6 mL) and stirred vigorously to obtain a homogenous mixture. Before precipitation, solution B had a pH of 11.2. After the addition of solution A, the fast precipitation of ACP made the pH value drop down to 7.6. After freezing and lyophilizing (Telstar LyoQuest 55 Eco), the powder was recovered and stored at 4 °C. Multiple batches were prepared to produce the amount of U-ACP nanofertilizers needed to be supplied in the pot-experiment. Each batch of nanoparticles was characterized by XRD, to certify the amorphous nature of the material and the absence of contaminants (inorganic salts), and by Fourier transform Infrared Spectroscopy (FTIR) to confirm the presence of both nitrate ions and urea molecules in the U-ACP nanoparticles. The chemical composition of powdered samples was analyzed by ICP-OES (Perkin Elmer OPTIMA 8300, Waltham, MA, USA). Moreover, 20 mg of the powdered sample were dissolved in 2 mL of ultrapure nitric acid and then diluted to 100 mL with Milli-Q water. The emission wavelengths were 317.93 nm (Ca), 213.62 nm (P) and 766.49 nm (K). The XRD data were collected on a Rigaku Miniflex 300 diffractometer using Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$), from 5° to 55° (2 θ) with a step size of 0.02° and scanning rate of 1.0° min⁻¹. Likewise, 2 mg of the sample were mixed with 150 mg of KBr and pressed by a hydraulic press (Specac, 2 tons, Orpington, UK) and FTIR spectra were collected with a spectral resolution of 2 cm⁻¹ by accumulating 32 scans in the 4000–450 cm⁻¹ range. Finally, the different batches of nanoparticles were mixed and homogenized. The nitrogen content in the nanoparticles (N: 6.43% (w/w)) was quantified by elemental analysis on a Perkin Elmer 2400 series II instrument (Waltham, MA, USA). Transmission electron microscopy (TEM) images were collected with a LIBRA 120 PLUS instrument (Carl Zeiss SMT, Centre for Scientific Instrumentation of the University of Granada (CIC-UGR), Oberkochen, Germany), operating at 120 kV. U-ACP nanoparticles collected by centrifugation were ultrasonically dispersed in ethanol, and then, a few drops of the slurry were deposited on 200 mesh copper grids covered with thin amorphous carbon films.

2.2. Plant Material and Fertilization Treatments

The trial was conducted in 2019–2020 in the experimental farm of the Research Centre for Viticulture and Oenology (CREA-VE), in Conegliano, Italy (45°51' N, 12°15' E), (Figure 1). Seven-year-old *Vitis vinifera* L. cv Pinot Gris grafted onto Kober 5BB rootstock were used for the experiment. Plants were grown outdoors under natural light and temperature conditions, in 80 L pots filled with a sand–peat–clay mixture (50–35–15% in volume). Sixteen vines with similar trunk diameter were selected during winter and cane pruned with 12 buds. Pots were positioned in rows with a spacing of 1 m between vines and 1.5 m between rows. Four fertilization treatments were applied, within a completely randomized design with four vines per treatment: N1: commercial granular NH₄NO₃ fertilizer (27%) at a dose of 45 kg N ha⁻¹ yr⁻¹, applied to the soil two times between budding and veraison and one in post-harvest. N dosage was calculated estimating the vine requirements [37–39] to supply the minimum N dosage to obtain the maximum allowed production (18 t ha⁻¹) and quality levels for Pinot gris in the Veneto area. The amount is consistent with the conventional N fertilization practice in northern Italy, where averages between 40 and 80 kg N ha⁻¹ are applied annually [40,41]. N2: U-ACP applied in fertigation at a dose of 36 kg N ha⁻¹ yr⁻¹ (the total amount of N was reduced by 20% compared to the conventional practice). An aqueous suspension of U-ACP (47 g L⁻¹) was applied to the soil three times between budding and veraison and one in post-harvest. N3: granular fertilization + foliar U-ACP. A total amount of 36 kg N ha⁻¹ yr⁻¹ was applied as follows: one application as granular NH₄NO₃ to the soil after budding, two foliar applications of an aqueous suspension of U-ACP (47 g L⁻¹) between flowering and veraison and one in post-harvest. As for N2, the total amount of N was reduced by 20% compared to the conventional practice. C: control, receiving no N fertilization. For all treatments 80% of the annual N was supplied between spring and early summer and 20% in autumn. A summary of the fertilization treatments is reported in Table 1. Plants of all treatments received equal amounts of granular P and K fertilizers (40 kg P₂O₅ and 80 kg K₂O ha⁻¹ yr⁻¹) and were

well watered throughout the vegetative seasons by an automatic drip irrigation system. Standard viticultural practices were applied for disease control.



Figure 1. Location of the experimental site at the Research Centre for Viticulture and Oenology in Conegliano, Veneto Region, Italy.

Table 1. Summary table of the fertilization treatments applied in the trial, with indication of the N source, the application method and the total amount of N applied per year.

Fertilization Treatment	N Source	Application Method	Tot kg N ha ⁻¹ yr ⁻¹
C	None	-	0
N1	Conventional fertilizer NH ₄ NO ₃	Soil application	45
N2	U-ACP	Fertigation	36
N3	Conventional fertilizer NH ₄ NO ₃ + U-ACP	Soil application + Foliar application	36

2.3. Climate

Weather data (temperature, air moisture and rainfall) were monitored with a weather station installed within the experimental site. Data readings were collected every 60 min and stored in a data logger (Watch Dog 1400; Spectrum Technologies, Bridgend, UK) for further analysis.

2.4. Leaf Chlorophyll Content

Leaf chlorophyll content was measured by using a portable Minolta SPAD-502 (Konica-Minolta, Osaka, Japan) chlorophyll meter at two time points in the two study seasons, flowering and veraison, as an indicator of the vine nitrogen nutritional status. Measures were taken on 8 fully expanded leaves per vine, inserted opposite to the basal bunches on main shoots.

2.5. Yield, Yield Components, and Grape Analysis

Grapes from all treatments were harvested at technological maturity, defined as total soluble solids (TSS) ≥ 18 Brix and titratable acidity (TA) ≤ 9 g L⁻¹ for Pinot gris in the local conditions. Yield per vine and average cluster weight were recorded using a hanging scale (CH, Kern, Germany). Grape composition was analyzed on four replicates of 60 berries per treatment, collected separately from each vine. Berries were weighed and then crushed for soluble solids and titratable acidity analysis on musts. Soluble solids were measured by refractometer (Atago PR32) at 20 °C. Titratable acidity (expressed as g L⁻¹ of tartaric acid equivalents) was determined using a Micro TT 2022 automatic titrator (Crison, Barcelona,

Spain) by titration with 0.1N NaOH. Yeast-assimilable nitrogen (YAN) was determined following the method described by [42]. Approximately 1 kg of grape from each replicate was stored at $-20\text{ }^{\circ}\text{C}$ for the fingerprint of the volatile compounds.

2.6. Determination of the Grape Volatile Compounds

The volatile compounds of the grapes were determined using static Headspace Solid Phase Microextraction (HS-SPME) coupled with Gas Chromatography/Mass Spectrometry (GC/MS) as previously described [43]. Samples were analyzed following a random sequence, in order to avoid biases. Briefly, for each replicate 8 g of berries were crushed and then transferred into a 20 mL vial. Afterwards, 5 μL of 4-methyl-2-pentanol internal standard (stock solution prepared diluting 50 μL of I.S. to 10 mL of Milli-Q water) solution was added. Furthermore, 0.5 mL of a saturated solution of NaCl and 1.5 g of citric acid were added into each vial, before sealing with a perforable screw cap.

Each sample was kept in a heating bath at $70\text{ }^{\circ}\text{C}$ for 2 h with continuous stirring at 250 rpm. Then, the samples were extracted by headspace-solid phase microextraction (HS-SPME) with a triphasic fiber (DVB/CAR/PDMS, 50/30 μm , 1 cm), which was inserted into the 20 mL vials by piercing of the septum. The fiber was therefore exposed to the headspace under a continuous heating at $70\text{ }^{\circ}\text{C}$ and stirring at 250 rpm. After an exposure time of 30 min, the fiber was removed from the vial and introduced into the injector of the gas chromatograph. All samples were prepared and immediately analyzed following a random order with respect to the study treatments, in order to avoid systematic errors.

The GC/MS analysis was performed with manual injection on an Agilent 7890A gas chromatograph coupled to an Agilent 5975 quadrupole mass detector, with thermal desorption at $240\text{ }^{\circ}\text{C}$ (temperature of the split/splitless inlet). The gas-chromatographic separation was carried with helium as the mobile phase on a MEGA-WAX Spirit column (0.30 μm /0.18 mm/40 m) in split mode (1:10). The flow rate applied was 0.7 mg/L. The oven temperature program was as follows: $40\text{ }^{\circ}\text{C}$ for 0.2 min; 40 to $180\text{ }^{\circ}\text{C}$ with at $3\text{ }^{\circ}\text{C}/\text{min}$ rate; $180\text{--}230\text{ }^{\circ}\text{C}$ at a $10\text{ }^{\circ}\text{C}/\text{min}$ rate; $230\text{ }^{\circ}\text{C}$ for 3 min. The MS analysis was performed on a quadrupole mass spectrometer applying a source electron ionization (EI) energy of 70 eV. The m/z range applied for analysis was 34–360 m/z and the scan rate was 1 spectrum/s. The ion source temperature was programmed at $230\text{ }^{\circ}\text{C}$ and the quadrupole as $150\text{ }^{\circ}\text{C}$.

The volatile compounds were identified by comparing the calculated linear retention index (LRI) and their mass spectrum with that reported in NIST 2007 data bank (Library and Chemistry Webbook) [44]. The LRIs were calculated according to the C5-C40 standard alkanes elution series (Sigma-Aldrich) separately injected. The LRI formula used for the calculation was according to Van den Dool and Kratz's reference [45].

2.7. Statistical Analysis

Yield components, grape composition and SPAD data were analyzed with the one-way analysis of variance (ANOVA). For every year of study, four replicates per treatment were used for all parameters. In case of significance of F test, mean separation was performed by the Tukey test, using STATISTICA 8 (StatSoft Inc., Tulsa, OK, USA). Principal Component Analysis (PCA) was carried out using the *prcomp* function implemented in the ggfortify package for R [46].

In order to further validate the results obtained with PCA, a cluster analysis was also run calling the *hclust* function, using the Ward's method with Euclidean distances. The experimental data were visualized and analyzed by using ggplot2 [47], Agricolae v.1.3-1 [48], and ggfortify [46] package within the R environment [49].

3. Results

3.1. Production and Characterization of U-ACP Nanofertilizers

The U-ACP nanoparticles were synthesized by chemical precipitation in presence of both urea and nitrate ions as N-dopants. The X-Ray Powder diffractogram of the resulting nanoparticles (Figure 2a) shows the typical trace of amorphous calcium phosphate.

The absence of Bragg peaks confirms the purity of the U-ACP material and discards the precipitation of any crystalline phase [30]. The FTIR spectra of U-ACP nanoparticles (Figure 2c) shows the typical vibration bands related to phosphate groups in amorphous calcium phosphate ($500\text{--}630\text{ cm}^{-1}$ and $1000\text{--}1200\text{ cm}^{-1}$) [50]. The sharp band at 1385 cm^{-1} is assigned to the antisymmetric stretching ν_3 mode of nitrate groups [51]. The bands at 1680 cm^{-1} and 1465 cm^{-1} are assigned to the characteristic bands of urea ($\delta_s(\text{NH}_2)$ and $\nu_{\text{as}}(\text{C-N})$ stretching, respectively) [52]. The nitrogen content of the U-ACP nanoparticles was determined by elemental analysis (N-content = 6.43 weight %). The amount of residual K, determined by ICP-OES, was $0.68 \pm 0.05\%$, that is nearly 30 times smaller (if the molar ratio is considered) than the nitrogen content in the material.

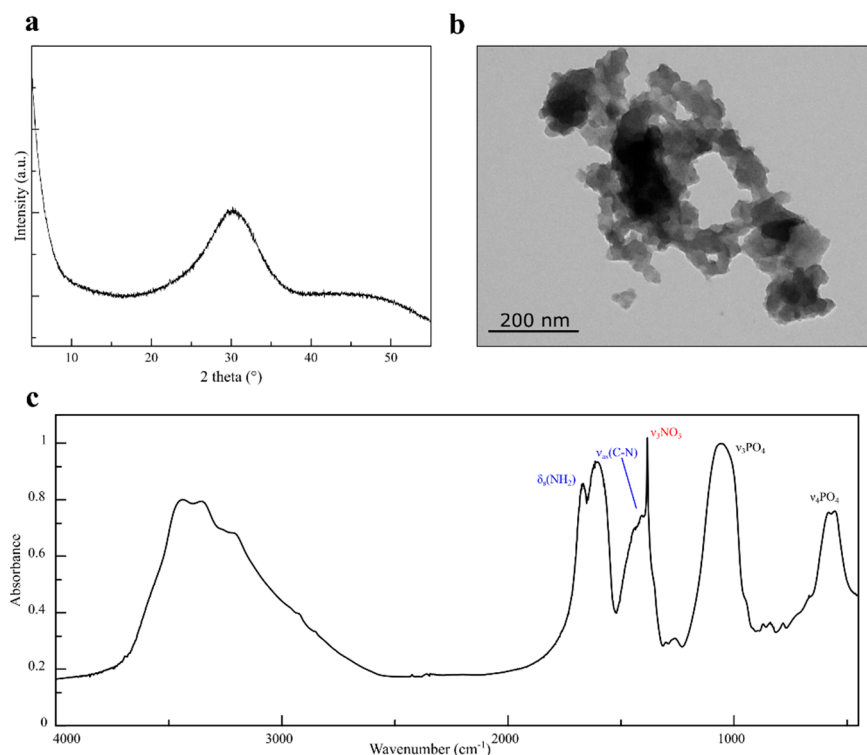


Figure 2. XRD diffraction trace (a), TEM image (b), and FTIR spectrum (c) of U-ACP nanoparticles confirming the successful functionalization of ACP with urea and nitrate. Vibrational modes of both N-containing dopants are depicted in blue and red respectively.

The principal chemical, structural, morphological and analytical properties making this ACP-based material different from ubiquitous nano-apatite were extensively discussed in previous studies [27,29,30] and are here summarized: (a) TEM imaging shows the presence of irregularly shaped nanoparticles, which are aggregates of smaller particles with sizes as low as 10 nm (Figure 2b); these values were confirmed by independent SAXS experiments; (b) neither diffraction peaks attributable to nanosized apatite or to other nanocrystalline calcium phosphates were present, nor did the XRD traces show the presence of residual crystalline urea or of inorganic salts as contaminants; only the typical broad diffraction halo was constantly found if the defined synthetic protocol was carefully followed; (c) ICP analysis provided Ca:P molar ratios near 1.92 ± 0.02 , a value higher than 1.66 (as expected for hydroxyapatite), but still in the range observed for ACP, where (hydr)oxo and carbonate anions might be present [53] (d) ICP and EDX chemical analyses, urea, calcium and phosphate release kinetics, surface, adsorbed or intergrain trapped urea, were studied and discussed in [27,30].

3.2. Climate

The 2019 and 2020 growing seasons were quite similar and typical of the area (Table 2). The first season was on average slightly warmer, with a total heat accumulation (Growing Degree Days) in the period 1 April–31 October, 1970 DD, compared to 1898 DD in the second one. Mean and minimum temperatures were slightly higher in 2019, while maximum temperatures and rainfall were almost coincident for the two years of study. The year 2020 was characterized by slightly lower minimum and higher maximum temperatures in the last phase of the ripening (August), resulting in higher diurnal thermal ranges in the weeks prior to the harvest compared to 2019.

Table 2. Climate data in the experimental site during the growing season (1 April–31 October) in 2019 and 2020.

Year	GDD ₁₀	T Avg (°C)	T Max (°C)	T Min (°C)	Σ Rainfall (mm)
2019	1970	19.2	24.8	13.9	843
2020	1898	18.8	24.8	13.0	839

3.3. Leaf Chlorophyll Content

The leaf N content, as estimated through the chlorophyll content of the leaves (SPAD), was measured at two stages during the two growing seasons: flowering and veraison. SPAD values were fairly similar in the two years, ranging between 25.5 and 32.9 for all treatments (Figure 3). A slight decrease over the growing season in both years was measured for all treatments, with average values ranging between 29 and 32 in flowering to 25.5 and 30.5 in veraison.

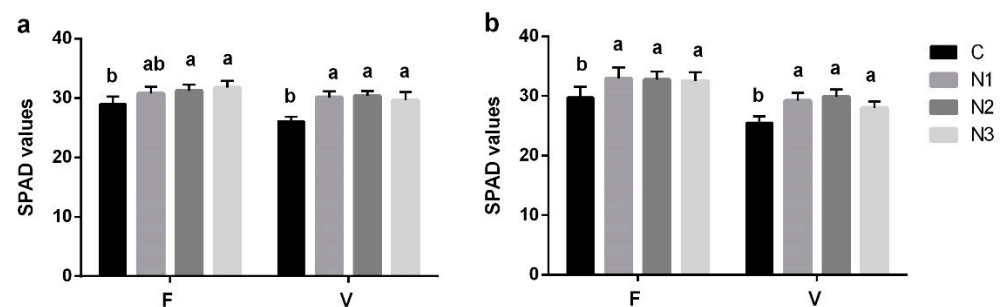


Figure 3. SPAD values recorded at flowering (F) and veraison (V) developmental stages for all treatments in the years 2019 (a) and 2020 (b). Vertical bars indicate standard errors (SE). Means followed by different letters differ significantly, as calculated using Tukey statistical analysis ($p \leq 0.05$).

We found significant differences only between the treatments receiving N and the non-fertilized control, whose SPAD values were always lower at all measuring dates. No differences were found between fertilized treatments, despite the lower N amount applied by nanoparticles (N2 and N3) compared to the commercial fertilizer (N1). The cumulated N amount applied before flowering was 13 kg lower for N2 and N3 compared to N1 (Supplementary Figure S1); at veraison, the cumulated N supply was 7.2 kg lower in the nanofertilizer treatments (28.8 kg for N2 and N3, and 36 kg N for N1).

3.4. Yield, Yield Components and Must Quality

Table 3 shows the yields and quality parameters of the musts for the years 2019, 2020, and the 2-year average for the different treatments. No significant differences were detected between the three N fertilized treatments (N1, N2, N3) for vine yield and yield components (bunch number, bunch weight, berry weight). As expected, the non-fertilized control displayed the lowest yield in both years, mainly attributed to a lower bunch weight. As regards the must quality, significant differences were found only in 2020 for TTS and YAN. In both years the highest TTS values were recorded in the control, which always

displayed the lowest yield. On the contrary, the lowest TSS values were always recorded in the N1 treatment, which showed the highest yield in both years of study. No differences were found among treatments as regards the titratable acidity. The YAN contents were similar among the three N fertilized treatments, which showed slightly higher values than the control in both seasons.

Table 3. Grapevine yield components and must quality parameters in the two years of study 2019 and 2020 for the four fertilization treatments. Data were processed using ANOVA; means followed by different letters differ significantly, as calculated using Tukey statistical analysis ($p \leq 0.05$).

PARAMETER	2019				2020				Average 2019–2020			
	C	N1	N2	N3	C	N1	N2	N3	C	N1	N2	N3
Yield (kg/vine)	2,0 b	2,6 a	2,3 ab	2,5 a	1,5 b	2,4 a	2,1 a	1,7 ab	1,8 b	2,5 a	2,2 a	2,1 a
Berry weight (g)	1,3	1,2	1,3	1,3	1,2	1,4	1,3	1,3	1,3	1,3	1,3	1,3
Number of bunches	27	28	27	32	21	23	20	19	24	26	24	26
Bunch weight (g)	75 b	96 a	83 a	79 ab	73 b	102 a	107 a	97 a	74 b	99 a	95 a	88 ab
TSS (Brix)	21,9	21	21,7	21,6	20,1 a	18,7 b	19,2 ab	20,0 a	21,0	19,8	20,5	20,8
Titratable acidity (g L ⁻¹)	8,3	7,8	8,3	7,7	5,7	6,3	6,5	5,7	7,0	7,1	7,4	6,7
YAN (mg L ⁻¹)	43,2	51,4	67,3	60,2	73,6 b	78,5 ab	112,6 a	85,9 ab	58,4	65,0	90,0	73,1

N1: granular NH₄NO₃ applied to the soil; N2: U-ACP applied to the soil; N3: granular NH₄NO₃ to the soil + foliar U-ACP; C: control with no N fertilization).

3.5. Grape Volatile Compounds

The volatile profile of crushed grape berries was determined in both years considered for the study (2019 and 2020). The GC–MS analyses allowed the identification of 22 volatile compounds among which there were alkyl- and benzyl- alcohols, aldehydes, ketones, and carboxylic acids present, which account for different aroma descriptors (Supplementary Table S1). The datasets collected in 2019 and 2020 were analyzed separately by multivariate pattern recognition analysis, i.e., principal component analysis (PCA). When the 2020 dataset was considered, the PCA highlighted a five components model describing up to 80% of the total variance. The scatterplot obtained by combining principal component 1 (PC1) and PC2 accounted for 51.02% of the total variance of the dataset, yet it did not display a separation of the samples according to the type of fertilization (Figure 4A). These results suggested that the volatile profile of berries was not significantly affected by the treatments, at least in the present experimental conditions. These observations were further confirmed by Hierarchical Cluster Analysis carried out on the same analytical dataset (Supplementary Figure S2). Interestingly, equivalent results were also obtained by analyzing the volatile compounds dataset obtained from berries sampled at harvest in productive season 2019 (Figure 4B).

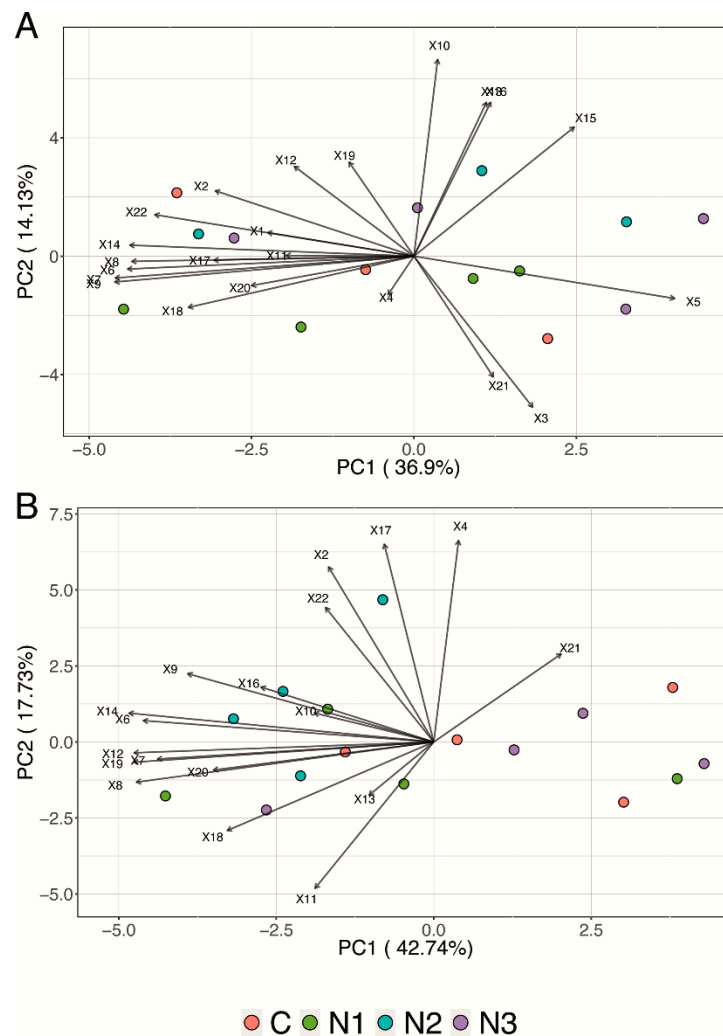


Figure 4. Principal component analysis (PCA) of the volatile compounds dataset. The two scatterplots represent the modification of Pinot gris berries volatile profile harvested from plants subjected to different fertilization practices (N1: granular NH_4NO_3 applied to the soil; N2: U-ACP applied to the soil; N3: granular NH_4NO_3 to the soil + foliar U-ACP; C: control with no N fertilization) in the production years 2020 (A) and 2019 (B). X1–X22, volatile compounds are described in Supplementary Table S1.

4. Discussion

In the context of a more sustainable and modern viticulture, the development of new forms of fertilizers based on innovative nanotechnologies is regarded as one of the promising approaches to significantly enhance the grape yield and quality, minimizing concurrently the environmental issues connected with the conventional fertilization [32,33]. In this study, we investigated the possibility of using urea-doped calcium phosphate nanoparticles (U-ACP) as nitrogen source for grapevine plants. The purpose was pursued by analyzing in plants fed with U-ACP nanoparticles the levels of yield and its quality in comparison with grapevines treated with conventional fertilizers. Particular attention was paid to the levels of N supplied in order to highlight the use-efficiency levels of this new form of fertilizer in comparison to the traditional one. In this respect, it should be highlighted that to date the scientific knowledge about the use of U-ACP in viticulture is very limited. In fact, only recently it has been reported the possibility to apply foliarly U-ACP to vine canopy at the veraison stage in order to improve the composition and the organoleptic profile of the grape [33]. It is clear that in this case the U-ACP application has been conceived and planned as a complement to the classical fertilization of the vineyard.

For this reason, information on the effect of U-ACP on grape yields and quality when they are used as an alternative to conventional fertilization techniques is still missing.

In this study, U-ACP application was tested using two methods to deliver nitrogen to the plant: fertigation (N2 treatment), which allows to tune the timing and the levels of nutrients' availability during the growing season [54,55], and foliar application (N3 treatment), which has already showed interesting effects in improving some qualitative properties in grapes [56,57]. Since an increased nutritional efficiency for U-ACP treatments was expected, the total amount of N applied in N2 and N3 was reduced by 20% compared to the conventional fertilization practice (N1).

In order to evaluate the effect of the treatments on the nutritional status of the plant during the growing season, the chlorophyll contents of the leaves (SPAD) at two key stages (flowering and veraison) were measured. In this regard it is important to highlight that SPAD indexes are considered a good indicator of the N content in leaf tissues [58–61] as a consequence of the N present in the chlorophyll molecules. Results here reported show that, as expected, SPAD values were totally different between fertilized and non-fertilized plants, regardless of the physiological phase considered. With respect to the fertilized plants, the average value of the SPAD index ranged between 28 and 33 without significant differences between the conventional fertilization and the U-ACP-based treatments (Figure 2). It is interesting to note that these values are in agreement with those reported by Vrignon-Brenas et al. [60] in potted Sauvignon blanc vines fertilized with conventional sources at annual rates (40 U) comparable to those used in this study. In other varieties, similar SPAD values were correlated to leaf N concentration levels between 1.8–2.5% [61], and even though this relationship is not perfectly similar for all cultivar, this SPAD values suggest an optimal N content for all our fertilization treatments, despite the lower N amount applied by nanoparticles compared to the conventional fertilizer.

Considering the grape production, in both years of this study the levels of yield per plant and yield parameters (i.e., bunch number, bunch weight, berry weight) were comparable between the treatments with nanoparticles and conventional fertilizer (Table 2). On the contrary, as expected, the levels of these parameters were significantly restrained when no nitrogen source was applied (control plants). In this respect it is well known that vine growth is often limited in the natural environment by low nitrogen availability. Despite the low N requirement of grapevines, N restriction reduces the annual biomass production and hence the final yield in comparison to vines supplied with optimal rates of N [62,63]. Previous studies on grapevine have shown that N availability can significantly influence berry set and floral bud initiation [62,64], parameters that are directly related to the final yield. This finding may explain the decreased bunch weight and the related lower yield observed in the non-fertilized control vines. Comparing the nano-fertilized and conventional treatments, despite the lower amount of N supplied with U-ACP, bunch weights were similar, suggesting a higher N use efficiency for the nano-fertilization. In fact, U-ACP display a high ability in incorporating foreign ions, a high adsorption capacity and solubility [27]. These factors enable a gradual release of nitrogen after the fertilizer application and facilitate its delivery and absorption by the plant. This likely results in an increased nutritional efficiency for plants which compensates for the reduction of applied nitrogen. Our results are in agreement with those obtained in previous tests where U-ACP were applied on durum wheat [27]. In fact, the number of fertile florets and the proportion of those setting grains in wheat plants treated with nanoparticles at reduced nitrogen dosages (by 40%) were unaltered in comparison to those conventionally fertilized and consequently, yields at harvest were similar.

Vineyard N management can also affect N accumulation in fruits and has significant consequences on berry composition and on the winemaking process [65,66]. Indeed, by promoting yields and vigor, N supply can affect the bunch microclimate, delaying berry maturity and influencing the sugar and acid content [67]. In our study, the fertilization with U-ACP lead to a must composition similar or even improved compared to that of the conventional fertilization (Table 3). In fact, TTS showed slightly higher values for the

nano-fertilized treatment (N2 and N3) with respect to the conventional one (N1), even if significant differences were found only in 2020. In both years the titratable acidity at harvest was comparable in all treatment. It has been reported that N supply may extend the vegetative growth delaying berry maturity [67]. However, in our study the conventional and nano-fertilization showed acidity levels similar to the control, suggesting that the rates applied did not affect either the ripening timing or the grape maturation trends.

It is well known that N supply can also affect the amount of yeast-assimilable nitrogen (YAN) in the must, which is a critical parameter since it controls the fermentation kinetics. In fact, high levels of YAN result in too rapid fermentation with the good chance to develop in the wines undesirable compounds. On the contrary, low levels of YAN are often associated with stuck or sluggish fermentations and hydrogen sulfide production [65,68]. In our study all the N fertilized treatments displayed YAN levels between 50 and 110 mg L⁻¹, which are in a low-range considering the minimum values reported in literature for optimum fermentation kinetics (approx. >100 mg N L⁻¹) [65]. No significant differences in YAN concentration were found between the nano-treatments and the conventional fertilization. These results are in agreement with those reported in Tempranillo [33], as they found that foliar application of U-ACP resulted in YAN levels similar to those of vine treated with urea at greater dose.

As nitrogen availability has an influence in the formation of numerous compounds involved in the aroma matrix of wine [56,66,69], grape volatile profiles have been evaluated for all the treatments here considered. These profiles, analyzed with different multivariate statistical elaborations (PCA and HCA), did not show any specific clustering of the four replicated samples representing the four treatments. The presence of a pretty similar aromatic profile among the treatments here found is in agreement with what described in Tempranillo grapes, where the N fertilization with proline, urea, and two commercial nitrogen fertilizers did not lead to an increase of grape primary aromas, such as terpenoids and norisoprenoids [56]. These observations therefore suggest that the novel fertilization strategies based on U-ACP are equivalent to the conventional ones, at least considering the qualitative features of grapes.

Concurrently to the assessment of the effect of U-ACP nanoparticles on grape yields and quality, another goal perused in this study was to assess the efficacy of the application approach (foliarly or to the soil) of the U-ACP nanoparticles. Preliminary studies in wheat plants showed that the U-ACP nanoparticles uptake takes place through both the leaf stomata and the epidermis of the roots, with the latter being much faster than through the stomata [27]. In our experiment, we compared two methods of application, fertigation and foliar spray. Fertigation permits an efficient application of fertilizer directly to the vine root zone and being applied in water solution makes root uptake independent from soil water content [70]. Foliar application, combined to a conventional fertilization in the first growing stages when leaf surface is still limited, represents an effective technique that ensures an efficient assimilation of N by the vine, and contribute to a more sustainable eco-friendly agriculture [56,71]. It has been also reported that foliar application of nitrogen can increase the grape amino acid content to a greater extent than that observed in the case of soil application [57]. Comparing fertigation and foliar application (N2 and N3 treatments, respectively) we did not observe significant differences for the whole yield or grape metabolic profile at harvest. While in other studies improvements in YAN concentration and aromatic compound contents were observed after foliar N supply [56,66,69], in this trial U-ACP application in fertigation and foliar spray were comparable for all the quality parameters analyzed (Table 2). This can be explained by the fact that, in most of the previous studies, N foliar supply was performed at veraison phase and usually repeated after this stage with the aim of improving grape composition. However, in our study, foliar N was supplied only until veraison stage, likely resulting in a smaller effect on grape composition at harvest. Both foliar and fertigation treatments provided production and quality levels comparable or even improved compared to those of the conventional fertilization. These results demonstrate that U-ACP nanofertilizers can efficiently deliver

nitrogen through the leaf stomata and the soil-root system, and in both ways their use can significantly reduce the N rates while maintaining yield and quality.

A final consideration must be addressed to the possibility of using U-ACP as multinutrient nanofertilizers. In fact, while this study was focused on the use of these nanoparticles as a sustainable alternative to conventional N fertilizers, previous studies have proven the ability of these nanomaterials to deliver efficiently other physiologically relevant macronutrients, like Ca and P [27,30,72]. Further research is therefore needed to gain knowledge on the effectiveness of U-ACP also as a P or Ca source for grapevine fertilization.

5. Conclusions

In this study we investigated the possibility of using urea-doped nanoparticles as an alternative to conventional fertilizers in the management of the nitrogen nutrition of grapevines. Experiments carried out by using Pinot gris vines in semi-controlled conditions showed, with respect to nitrogen, a similar nutritional status of the plants when fed with nanoparticles or with a conventional fertilizer, despite the 20% lower level of total annual nitrogen supplied with nanoparticles. Similarly, yield and grape quality parameters (i.e., sugar content, titratable acidity, and aromatic content), were comparable among the plants treated with the different N sources (nanoparticles or conventional fertilizer). Moreover, the different approach of nanoparticles application (foliarly or to the soil) seems to not affect the efficiency in the use of this source by the vine plants.

Collectively the results here reported provide clear evidence of the efficacy of U-ACP nanoparticles as a nitrogen source for vine plants allowing also a restraint of the N dosage applied at the field scale. Moreover, they are the premises for the development of alternative nitrogen fertilization strategies, optimizing the dosage/benefit ratio and being of particular interest in a context of a more sustainable and modern viticulture.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11061026/s1>. Table S1: List of identified aromatic compounds, Figure S1: Cumulated amount of nitrogen applied to the vines of the four fertilization treatments, Figure S2: Hierarchical Cluster Analysis (HCA) of the aromatic compound dataset.

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Data Availability Statement: Data are contained within the article. Further details about the reagents used in this study are publicly accessible in the websites: www.alfenatura.com, www.myprotein.com, www.algin-chemie.de, www.buxtrade.de, accessed on 20 May 2021.

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