Clays and Clay Minerals

FIGHTING AGAINST PLANT SALINE STRESS: DEVELOPMENT OF A NOVEL BIOACTIVE COMPOSITE BASED ON BENTONITE AND L-PROLINE

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Abstract—Soil salinity is one of the most critical environmental stresses that affects crop productivity. In a context in which world demand for food is growing continuously, this problem requires urgent attention. Actions that go beyond traditional agricultural practices are needed. The objective of the current study was to develop a bioactive, economic, and sustainable compound that can increase the tolerance of cultivated plants in saline-stress situations by combining the hosting capacity of natural bentonite nanoclay (Bent) with a phytoactive osmoprotective compound, L-Proline (Pro). The Bent-Pro nanocomposite synthesis method, its final chemical structure, and in vitro bioactivity were addressed here. The results indicated that Bent can retain a maximum of 14.4% (w/w) of Pro. The (001) X-ray diffraction (XRD) peak of Bent shifted to smaller angles in the pattern of Bent-Pro, indicating that Pro has a monolayer arrangement between the Bent layers. The results of transmission electron microscopy (TEM) also supported this result. Pro was also retained on the edges or external surfaces of Bent, as indicated by thermogravimetric analysis (TGA) and scanning electron microscopy (SEM). In addition, Pro functional groups identified by Fourier-transform infrared (FTIR) spectroscopy indicated that it was present in its zwitterionic form. The role of Bent-Pro as a protector against plant saline stress was assayed using *Arabidopsis thaliana* (*A. thaliana*) as a model, demonstrating that it mitigates the detrimental effects of NaCl-mediated salt stress on seed germination and the leaf chlorophyll level, thus highlighting the relevance of this contribution and the versatility and broad applicability of clays.

Keywords——Bentonite · Biomaterial · L-proline · Plant acclimation · Salt stress · Zwitterion

INTRODUCTION

Phyllosilicates are nanostructured inorganic lamellar materials available widely in nature and usually referred to as nanoclays. They are layered aluminosilicates, which means that their crystalline structure is formed by Al and Si oxide sheets stacked on top of each other. The specific spatial organization of two, three, or up to four sheets of $[SiO_4]^{4-}$ and/or $[AlO_3(OH)_3]^{6-}$ leads to the formation of the different 1:1, 2:1, and 2:1:1 types of phyllosilicates (Pédro 1965). A clear representation of these nanoclay crystalline structures can be found in a previous study (Pédro 1965). Among them, 2:1 phyllosilicates are of particular importance as they include the smectite group, an expansible kind of clay, used widely due to its great versatility and multiple applications.

In line with this classification, the montmorillonite (Mnt) mineral belonging to the smectite group is the most commonly used nanoclay for chemical modification. It is the main component of bentonite (Bent), which is widely available in Argentina, having quartz and feldspar as impurities. The Bent layers present negative charges resulting from isomorphic substitutions which occur naturally and refer to the replacement of an element by a similar one without altering the mineral's chemical structure. The negative charges generated due to this natural process are compensated by incorporating inorganic cations such as Na^+, K^+, Mg^{2+}, and Ca^{2+} in the interlamellar space. These cations are also known as "exchangeable cations" as others, including organic cations, can replace them (McConnell 1950).

Naturally occurring clays are often modified to provide them with specific functions or properties (Pédro 1965). The idea of using nanosystems for the retention and release of active substances has been used widely in the fields of medicine and cosmetics (Patel et al. 2006; Ruiz-Hitzky et al. 2010), polymer science (Alexandre and Dubois 2000; Merino and Alvarez 2020; Merino et al. 2016; Wu et al. 2010), and, of late, has also been used in agriculture (Sanhez-Martin et al. 2006; Gamba et al. 2015; Merino et al. 2018, 2020; Mansilla et al. 2020). These carrier systems lead to greater application efficiency and less associated contamination. In general, these include the possibility of reducing the volume of the doses applied and offer

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protection against drug environmental degradation, evaporation, and leaching (Grillo et al. 2021).

In the context of the projected increase in crop production to meet the growing demand for food (~50% more by 2030 according to the Food and Agriculture Organization of the United Nations (2017)), agricultural nanotechnologies are urgently required. Nanoclays are inexpensive, non-toxic, abundant materials and offer the possibility of functionalization. The number of publications reporting their use for the controlled release of agrochemicals such as fertilizers, pesticides, herbicides, and biostimulants and fertilizers has increased over recent decades (e.g. Celis et al. 2002; Lagaly 2001; Li et al. 2008; Mansilla et al. 2020; Merino et al. 2018, 2020; Ni et al. 2011).

Soil salinity is one of the most critical environmental stresses that impacts the growing demand for food crops as the majority of the plant species consumed worldwide are susceptible to salt stress. The amount of irrigated land estimated to be affected by salinity is ~ 45 million hectares, resulting in ~ US$ 12 billion of annual losses in agricultural production (Negrão et al. 2016; Shahid et al. 2018). Salinity affects crops by limiting their ability to take up water, leading to poor germination and seedling establishment, in addition to Na^+ toxicity, which has detrimental effects in multiple plant-cell responses at the molecular, biochemical, and physiological levels (Golldack et al. 2014; Zhu 2002).

When exposed to salinity, many plants induce the accumulation of osmoprotectants or compatible solutes consisting of low-molecular-weight secondary metabolites such as the amino acid L-Proline (Pro). These osmoprotectants ameliorate osmotic stress by maintaining turgor pressure and plasma membrane ion efflux, and by controlling transpiration rate (Ashraf and Foolad 2007; Kaur and Asthir 2015). Stress-tolerant plant species, therefore, have been associated with greater concentrations of Pro compared to salt stress-sensitive plants. In addition to its role as a compatible osmolyte, Pro action has been associated with the stabilization of sub-cellular structures, the scavenging of harmful free radicals generated by the stress, and the modulation of gene expression (Per et al. 2017; Szabados and Savouré 2010). Given that the action of Pro in plants is highly sensitive to its application doses, it is essential to optimize the concentration and treatment time according to the requirements of the individual crops (Ashraf and Foolad 2007). The incorporation of Pro in Bent could offer advantages for its protection against possible biological degradation together with the administration of controlled doses and its sustained release over a certain period. Taking into account the potential of the exogenous application of Pro as a scavenger for reactive oxygen species (ROS) and also as an inductor of the antioxidative enzymes (Hayat et al. 2012), the support and carrying of Pro in a nanoclay would potentially improve the bioefficacy of the product against stressful environments on plant crops.

The objective of the current study was to develop a bioactive, economic, and sustainable compound that can increase the tolerance of cultivated plants to saline stress. The hypothesis presented here is that Bent nanoclay can act as a vehicle for an osmoprotective compound, such as Pro, and that this 'Bent-Pro' nanocomposite can contribute to maximizing the yield of cultures. *Arabidopsis thaliana* (*A. thaliana*) was used as a suitable model for studying responses to saline stress. Promising results were obtained, emphasizing the relevance of this contribution toward a sustainable future in agriculture and highlighting the versatility and broad applicability of clays.

MATERIALS AND METHODS

Materials

The clay used here was a bentonite obtained from Minarmco S.A. (Neuquén, Argentina), consisting of Mnt with quartz and feldspar as the main impurities and was used without further treatment (XRD data given in Fig. 1). The Bent CEC was determined by the methylene blue method to be 105 meq/100 g of clay (Merino et al. 2018). The amino acid Pro (≥ 99.0% purity) was acquired from Biopack (Buenos Aires, Argentina).

Preparation of Bent-Pro

Various syntheses were carried out in order to optimize the relative amounts of the reagents involved and the reaction times that enable the maximum retention capacity of Pro in Bent. For this, a 1 wt.% suspension of Bent in distilled water was kept under stirring, together with varied amounts of Pro for 2 h at 20°C. Then, the solutions were centrifuged at 10,733×g for 15 min. The supernatant was collected for the subsequent determination of Pro, and the modified nanoclay was washed three times with distilled water. The samples were frozen at –20°C and lyophilized at 40 Torr and −55°C for 72 h using a Columbia International freeze dryer (Irmo, South Carolina, USA) to obtain them in fine powder form. The resulting nanoclays were designated: Bent/0.5Pro2H, Bent/1.0Pro2H, Bent/1.5Pro2H, Bent/2.0Pro2H, Bent/4.0Pro2H, and Bent/8.0Pro2H, where the number preceding Pro is the CEC fraction (*f*) representing the amount of Pro used and 2H indicates the reaction time, 2 h. The Pro mass to be incorporated, M_{Pro}, was calculated using Eq. 1:

$$M_{Pro} = f \cdot \ CEC \cdot X \cdot MM \cdot \ 10^{-3} \tag{1}$$

where the CEC is 105 meq/100 g of clay, X is the amount of clay to be used, and MW is the molecular weight of Pro (115.13 g/mol). Finally, the effect of reaction time on the amount of Pro retained by Bent was studied. For this, $f = 2$ was used, and times ranged from 0.5 to 8 h.

Pro is an amphoteric molecule and can, therefore, act as an acid or a base depending on the pH. This species can also be neutralized internally, forming a zwitterion. The isoelectric point of Pro occurs at pH 6.3 (McKee and McKee 2003); in the pH of the distilled water (~5.8, slightly acidic due to the effect produced by the dissolution of atmospheric CO_2) it would exist in an ionized form and with a slight prevalence of positive charge. In the present study, a decision was made not to reduce the pH further,

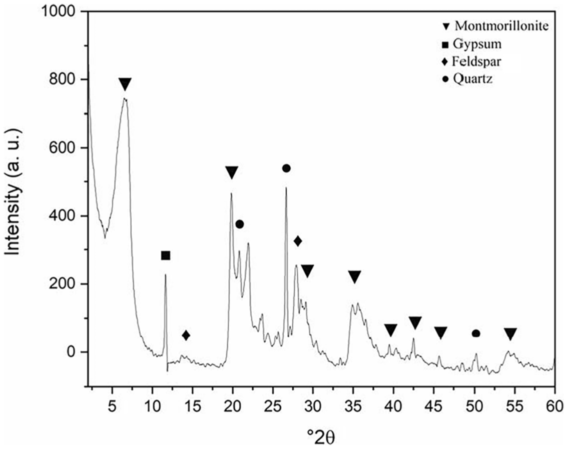

Fig. 1 XRD pattern of the natural bentonite

in order to increase the proportion of species with positive charge as it has been described previously that zwitterionic species can be intercalated in the Mnt interlayer space (Zhu et al. 2017). In addition, a strong acid medium could damage the structure of the clay (Komadel and Madejová 2006); furthermore, a new reagent included in the production process would add more cost.

Pro Quantification

The organic mass contents of Pro supported by Bent were determined using the method described by Bates et al. (1973), according to which 3 µL of the Bent-Pro synthesis supernatants was brought to a final volume of 300 µL using a 3 wt.% aqueous solution of sulfosalicylic acid (99% purity, Sigma-Aldrich, St. Louis, Missouri, USA). One volume of this fraction was mixed with equal volumes of glacial acetic acid (≥ 99.7% purity, JT Baker, Phillipsburg, New Jersey, USA) and a 2.5 wt.% solution of ninhydrin (99% purity, Sigma-Aldrich, St. Louis, Missouri, USA) in acetic acid and incubated at 100°C for 30 min. Subsequently, the absorbance at 540 nm was measured using an Ultrospec 1100 spectrophotometer (Amersham Biosciences, Amersham, UK), and the Pro content was determined from its calibration curve.

Characterization Techniques

FTIR. The FTIR spectra were measured by attenuated total reflectance (ATR) using a Perkin Elmer 100 infrared spectrophotometer (Akron, Ohio, USA), in the range 600–4000 cm^{-1}, with 16 scans at a resolution of 4 cm^{-1}.

XRD. The Bent and Bent-Pro powder samples were analyzed using a Malvern X-Pert Pro diffractometer (Malvern, UK), operating at 40 kV and 40 mA, with CuKα radiation ($\lambda = 1.54$ Å), at a scanning speed of 1º2θ/min and size of step of 0.02º2θ. The interlamellar spacings (d_{001} values) were calculated from the 2θ values using the Bragg equation (Eq. 2) (Bragg and Bragg 1913).

$$n\lambda = 2d \sin \theta \tag{2}$$

TEM. The shape, size, and arrangement of the Bent and Bent-Pro layers were investigated using a Jeol JEM 2100 (Tokyo, Japan) transmission electron microscope at a magnification of 150,000 ×. The operating voltage was 200 kV, and a B_6La filament was used. The sample was dispersed in acetone, and then a drop was placed on a perforated copper grid covered with carbon.

TGA. The TGA was performed using a TA Instrument HI-Res thermal analyzer (New Castle, Delaware, USA) at a heating rate of 10°C/min and from room temperature to 900°C in airflow. The mass of all the samples was in the range 20–30 mg. The degradation temperatures were obtained from the maximum of each event in the curves derived from TGA (DTGA).

SEM. The morphology of Bent and Bent-Pro nanoclays was analyzed under a FESEM Supra55 Zeiss microscope (Oberkochen, Germany) with an acceleration voltage of 3 kV. The samples were coated with a thin layer of gold in order to ensure electrical conduction.

Bioassays

Plant Material, Growth, and Treatments. A. thaliana seeds from the Columbia (Col-0) ecotype were surface sterilized in 30 vol.% of sodium hypochlorite (commercial concentration of 55 g of Cl/L, Ayudín, Clorox Argentina SA, Buenos Aires, Argentina) and 0.2 vol.% of Tween-20 (> 99% purity, Sigma-Aldrich, St. Louis, Missouri, USA), for 10 min, followed by three washing steps with sterilized distilled water. The seeds were sowed under laminar flow on Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, Missouri, USA) with 0.8 wt.% agar (BD-Difco, Sparks, Maryland, USA) in Petri plates and stratified at 4°C for 2 – 3 days in the dark. Then, seeds were germinated in a growth chamber at 23°C under 250 µmol photons/m^{2}s, with 16:8 h light:dark cycles.

Germination Assay. Approximately 50 seeds per technical replicate per treatment were sown on plates containing 20 mL of MS medium supplemented with 150 mM NaCl (> 99.0% purity, JT Baker, Phillipsburg, New Jersey, USA) in combination with increasing concentrations of Bent-Pro (0.01, 0.1, and 1 mg/mL), or the respective concentrations of free Pro (based on ~ 10% of Pro incorporation in Bent-Pro nanosystem), as a reference of the active compound. Bent (1 mg/mL) was used as the negative control. After stratification, the seeds were transferred to the growth chamber for two days. The percentage of germination was scored according to seeds in the 0.5 stage (Boyes et al. 2001). Four independent experiments with two replicates each ($n = 800$ seeds) were performed. The working NaCl concentration used for this assay (150 mM) corresponds to the concentration required for 50% inhibition of germination of wild-type seeds and was already set up in previous studies (Iglesias et al. 2010).

Chlorophyll Content. Ten-day-old seedlings grown on MS medium agar plates were transferred to 50 mL of liquid MS medium or MS containing 200 mM NaCl, supplemented with Bent-Pro (0.1 mg/mL), Pro (0.01 mg/mL), or Bent (0.1 mg/mL) for 2 days. Leaves (0.5 g) were ground in liquid N_2, and the powder was extracted with 80% acetone for 30 min in the dark. Plant extracts were centrifuged at $10{,}000 \times g$ for 20 min. The amounts of chlorophyll A and B were measured spectrophotometrically at 645 and 663 nm (Arnon 1949). Three independent experiments with two technical replicates each ($n = 240$ seedlings) were performed. The working NaCl concentration used for this assay (200 mM) corresponded to the condition required for control seedlings to show a 50% reduction in their chlorophyll content. These experimental conditions were established previously by Iglesias et al. (2010).

Statistical Analysis. The values shown in Figs. 7 and 8 are mean ± standard errors (SE) of the corresponding independent experiments. The data were subjected to analysis by the Student-t test against the control in *GraphPad Prism* version 5.01 software (*$p < 0.05$, **$p < 0.01$).

RESULTS AND DISCUSSION

Optimization of the Bent-Pro Synthesis Conditions

The adsorption of amino acids on clays depends on several conditions, including temperature, relative concentration among reactants, exchangeable cations present in the clay, type of clay, and pH (Petra et al. 2015). In the present study, the effects of various proportions of Bent and Pro and the reaction time under the conditions mentioned in the Materials and Methods section were studied.

First, the Bent-Pro nanocomposite preparation was studied at a fixed reaction time (2 h), and the effect of the initial amount of Pro, f, was studied. Values of $f = 0.5$, 1, 1.5, 2, 4, and 8 were studied, and the amount of Pro retained in Bent increased with increasing f until reaching $f = 2$ and then decreased with larger f values (Fig. 2). In general, the amount of Pro incorporated was slightly greater than the CEC of the clay, and excess Pro failed to produce significant increases in the amount retained. The results found here were superior to those reported by Petra et al. (2015) for the amino acids cysteine and glutamic acid loaded on Mnt. Those authors worked at pH 4, conditions that favor a cation exchange process, and thus limited the maximum amount of amino acids retained to the value of the CEC of the clay.

Next, the effect of reaction time on the incorporation of Pro in Bent was studied. For this, the variable f was set at 2, and the amount of Pro that was incorporated in Bent was studied as a function of reaction time: 0.5, 1, 1.5, 2, 3, and 4 h. The results showed that the amount of Pro retained increased with reaction time until 2 h was reached, where retaining ~ 1.2× the value of the CEC of the clay was

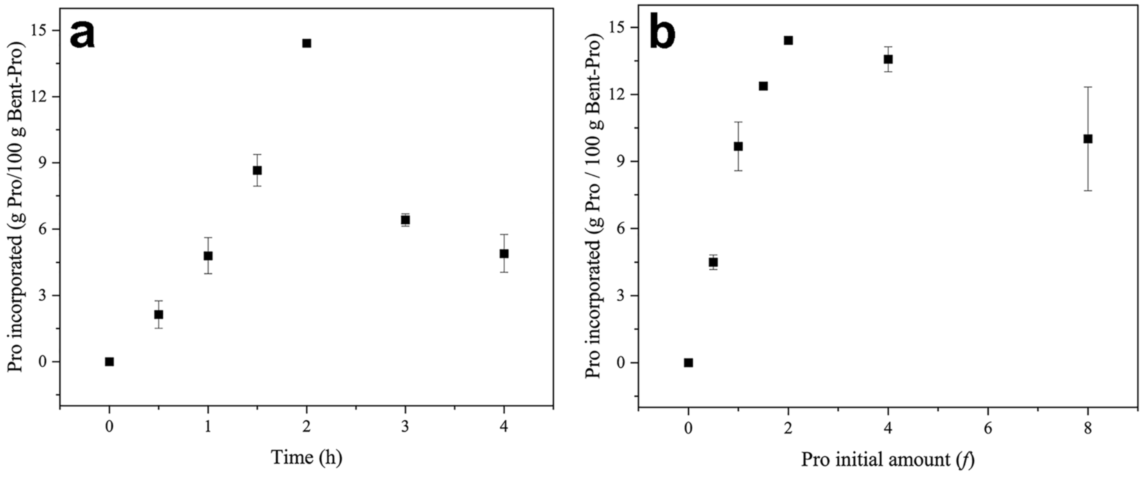

Fig. 2 Amount of Pro incorporated in Bent (g of Pro/100 g of Bent-Pro) depending on **a** the reaction time (h), and **b** the initial amount of Pro (f)

possible, which is equivalent to 14.4 wt.% of Pro. Longer times produced smaller amounts of Pro loading (Fig. 2).

At least two amino acid-adsorption mechanisms operate on smectites, such as Bent, according to Parbhakar et al. (2007). While cation-exchange mechanisms predominate at low amino acid concentrations and pH, adsorption at higher concentrations has been observed. In the present study, note that the pH of the reaction medium was not reduced and the predominant form of the Pro amino acid was zwitterionic. The observed effect as a function of time could, therefore, be related to the molecular rearrangements of Pro between the Bent layers. The loading of a given substance in Bent occurs as a process of 'self-assembly' governed by the following forces: (1) the electrostatic interaction between the protonated amino group of Pro and the internal and external surfaces of Bent; (2) the electrostatic repulsion between the carboxylate group of Pro and the Bent layers; (3) the electrostatic interaction between the interlamellar cations present in Bent and Pro; (4) the electrostatic interaction among Pro molecules; and (5) the interaction between all the compounds mentioned with the water molecules of the solvent. The way that these components are organized in space, resulting in the Bent-Pro nanoclay, is determined mainly by the combination of these factors, and will be further discussed below (Zhu et al. 2017).

Physicochemical Properties of Bent-Pro Nanoclay

The incorporation of Pro in Bent was reflected in new stretching bands in the FTIR spectrum of Bent. The new bands were centered at 1700 cm^{-1} (υ C=O), 1557.2 cm^{-1} (the NH_2^+ δ in-plane), and 1418.6 cm^{-1} (υ symmetric COO$^-$) (Devi et al. 2009). A reduction in the intensity of the absorption band centered at 3390.6 cm^{-1} (υ of OH and NH overlapping) and a shift of the stretching band of the Si–O group in Bent toward higher wavenumbers (Chen et al. 2018), from 996.1 to 1009.1 cm^{-1} (Fig. 3), were observed.

The stretching bands of COO$^-$ and NH_2^+ in the Bent-Pro spectrum indicated that the Pro molecules were intercalated in their zwitterionic form. The existence of a small peak at 1700 cm^{-1} could also indicate the presence of carboxylic acid groups and, consequently, a partial contribution of cation exchange. The isoelectric point of Pro occurs at pH 6.3; so at the pH of deionized water (~5.8) it has a small positive charge (McKee and McKee 2003). Nevertheless, the absence of other bands associated with carboxylic groups, such as OH stretching at 3500 cm^{-1}, and bending between 1200 and 1300 cm^{-1}, suggest that Pro was intercalated preferentially in its zwitterionic form.

The intercalation mechanism of a zwitterion between the Mnt layers was described previously by Zhu et al. (2017) who explained that the intercalation mechanism occurs in two stages: the first being the electrostatic interaction; and the second, a co-adsorption process that refers to a Van der Waals-type interaction process between the hydrophobic chains of the rest of the zwitterions (Zhu et al. 2017). Thus, specific rearrangements in time probably result in a reduced Pro in Bent retention when the reaction extends for > 2 h.

The Bent-Pro XRD pattern showed a shift in the position of the (001) diffraction peak (Fig. 4a), which is related to

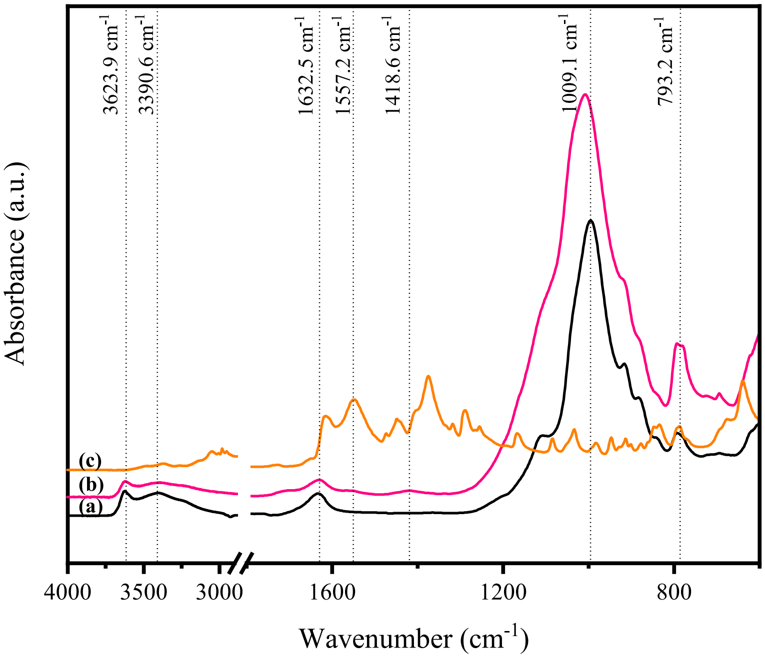

Fig. 3 FTIR spectra of **a** Bent, **b** Bent-Pro, and **c** Pro

the distance between the clay layers. Such shifting to lower angles (smaller values of 2θ) is related to an increase in interlamellar spacing and indicates that the amino acid is partially intercalated between the Bent layers (Patel et al. 2006).

The interlamellar spacing of Bent was 11.5 Å, and after the reaction with Pro, this parameter was increased by 3.4 Å, which indicated that some of the amino acid was incorporated in the interlamellar space. Similar results were found for the modification of Mnt with the amino acids valine, leucine, isoleucine, methionine (Mallakpour and Dinari 2011), and arginine (Shokri et al. 2017). On the other hand, the molecular size calculations carried out with the *Chem3D Pro* program showed an approximate value of 3.7 Å for the Pro molecule. The results indicated, therefore, that Pro would be oriented in a monolayer between Bent layers (Fig. 4b).

Transmission electron microscopy allowed direct observation of the changes produced in the interlamellar spacing

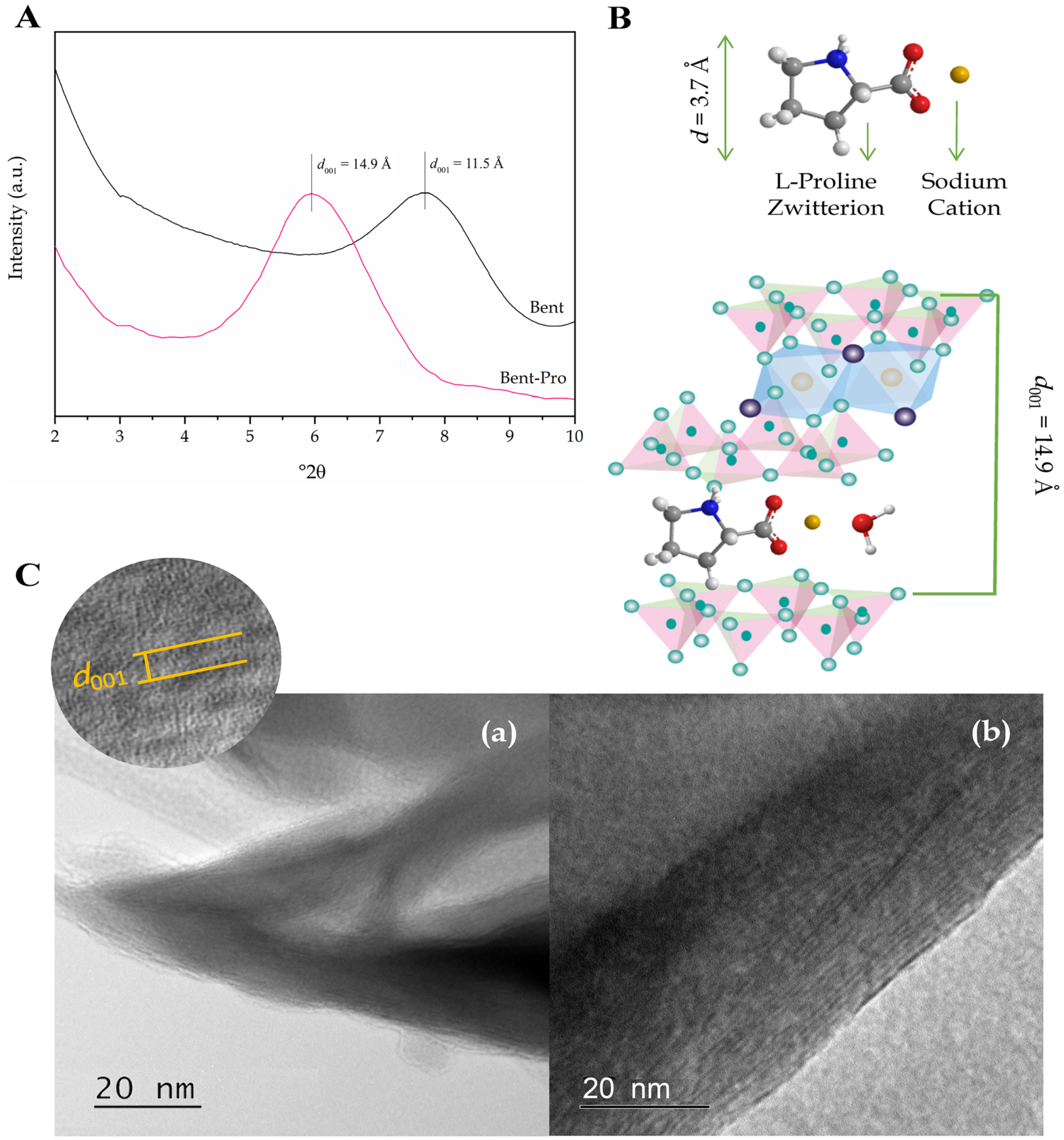

Fig. 4 **a** Bent and Bent-Pro XRD pattern between 2 and 10°2θ. **b** Representative scheme of the orientation of a Pro molecule (3.7 Å) confined in the interlamellar space of Bent, in its zwitterionic form, in the presence of a Na^+ cation and a water molecule; **c** TEM images of **a** Bent and **b** Bent-Pro

of the clay after its modification. Examination of the laminar structure of clays is not straightforward, however (Mallakpour and Dinari 2011). In general, it is believed that the high energy of the electron beam could remove the water of hydration of the cations present in the Bent interlayer space, causing the layers to collapse. The same might even be observed for the modified clays; the images needed to be captured quickly. The observed spacings were of the order of 11.8 Å for Bent and 13.9 Å for Bent-Pro (Fig. 4c), in agreement with the results observed by XRD.

The thermal stability of the Bent-Pro nanoclay and the mechanisms involved in its thermal degradation, which are related directly to their assembly, were analyzed by TGA.

The nanoclay Bent presented three events in the curve of DTGA (Fig. 5a) with maximum temperatures at 63, 110, and 624°C. The associated mass losses were 6.6, 0.9, and 3.6%, respectively. These events are related directly to the evaporation of slightly adsorbed water, water present in the interlayer space, and the dehydroxylation process that leads to the collapse of the interlayer of the clay, respectively, as previously reported (Mallakpour and Dinari 2011; Merino et al. 2016).

By incorporating Pro into Bent, significant changes were observed in the appearance of the DTGA curves, suggesting a smaller moisture content and the presence of organic matter (Mallakpour and Dinari 2011). Notably, three events were observed in the DTGA curve for Bent-Pro (Fig. 5b). The first was associated with the evaporation of slightly adsorbed water (49°C, 5.5%) with less interlamellar water than in Bent overlapped in this same event. According to previous publications, this observation agrees with the expected results after Bent loading with an organic compound (Mallakpour and Dinari 2011; Merino et al. 2016). Some of the water molecules in Bent were displaced during the intercalation of Pro and are more weakly bonded, demonstrating that Bent hydrophilicity was reduced slightly.

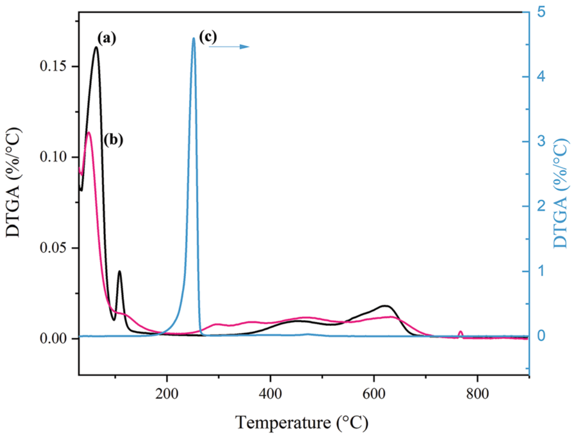

Fig. 5 DTGA (%/°C) vs. Temperature (°C) for **a** Bent, **b** Bent-Pro, and **c** Pro

The second event was related to the thermal degradation of Pro. It occurred in two stages associated with the maximum temperatures, 297.4 and 364.6°C, in overlapping events. The thermal analysis of free Pro (Fig. 5c) revealed a single maximum degradation step at 251°C, thus suggesting a strong interaction between Pro and Bent in the Bent-Pro nanocomposite. Because Pro can be found in three different spaces, namely, intercalated between the clay layers, adsorbed at the edges, or adsorbed on the external surfaces, it is expected to degrade over a wide temperature range (Mallakpour and Dinari 2011; Merino et al. 2016). As explained by Zhu et al. (2017), when the amount of compound to be intercalated exceeds the CEC, the surplus can be retained by Van der Waals type interactions, causing the appearance of a new peak in the DTGA at a lower temperature than that noticed for the intercalated Pro. In this way, the appearance of two temperatures of degradation for Pro in Bent-Pro could indicate that Pro is in at least two different environments and that some of the Pro retained is interacting by electrostatic forces, while the rest is interacting via weaker forces such as dipole–dipole or Van der Waals type. An estimation of the amount of Pro retained by Bent was also carried out by comparing the areas under the DTGA curves for Bent and Bent-Pro in the 200–800°C range, giving a 17% Pro intercalated, slightly more than the amount determined by the analytical method of Bates et al. (1973). Finally, the third event was attributed to the dehydroxylation of the clay with a maximum degradation rate at 631°C.

The morphology of the materials developed was observed by SEM. After loading Pro in Bent, the surface of Bent (Fig. 6a) initially smooth and compact, changed subtly to a more spongy and rough morphology in Bent-Pro (Fig. 6b), suggesting that some of the amino acid could be found on the edges or external surfaces of the clay (Merino et al. 2018), in line with the results from TGA analysis. These results are consistent with those of Chen et al. (2018) who observed a rougher surface after the intercalation of the amino acid cysteine into Bent.

Characterization of the Biological Action of Bent-Pro in the Protection of A. thaliana Against Salinity

Salinity affects the growth and production of plants by reducing the water potential of the soil solution, decreasing water availability. At the same time, an ionic imbalance is established, interfering with mineral nutrition and cellular metabolism. Multiple effects at various scales are possible, including reduction of turgency and growth, loss of cell structure due to membrane disorganization, and inhibition of enzymatic activity as the consequence of the combination of water stress, ionic toxicity, and nutritional imbalance (Saha et al. 2015). The most significant effects at physiological and biochemical levels, however, are the inhibition of

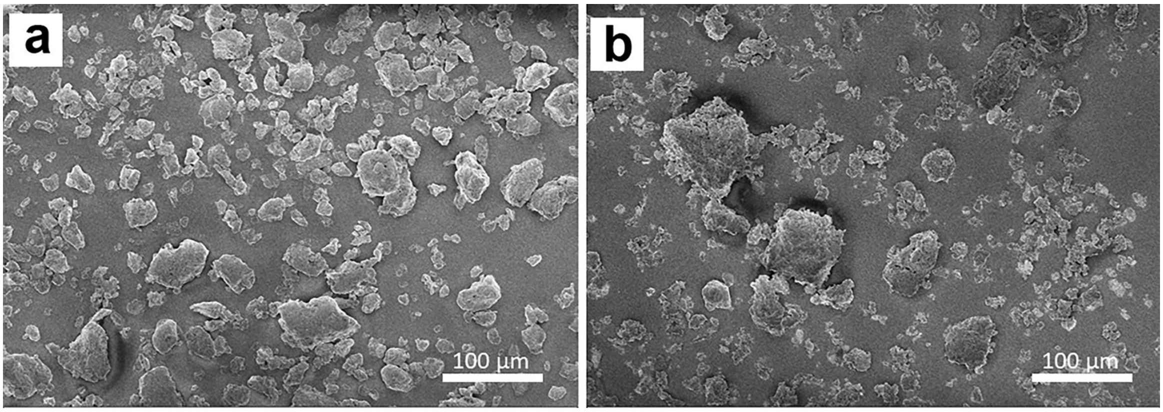

Fig. 6 SEM images of: **a** Bent and **b** Bent-Pro

seed germination and chlorosis (loss of photosynthetic pigments in leaves) (Saha et al. 2015).

In this context, the protective role of Bent-Pro nanoclays in the protection of the model plant *A. thaliana* against salinity was investigated. This plant species is sensitive to moderate levels of NaCl and has been suggested as a good model for the study of plant responses against salt stress (Zhang et al. 2004). Under salt-stress-free conditions, the germination rate of this species was ~ 98%. After a 48-h treatment on 150 mM NaCl-supplemented medium, the germination rate dropped to 32% (Fig. 7). Seeds sown in 150 mM NaCl in combination with increasing levels of Bent-Pro germinated at increasingly improved rates, reaching ~ 47% with a 0.1 mg/mL optimum dose of Bent-Pro, which was slightly greater than for equivalent doses of free Pro (Fig. 7).

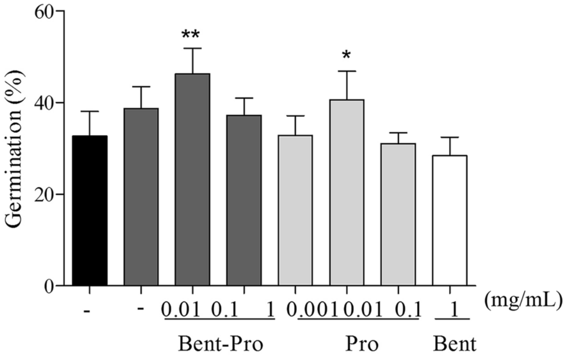

Fig. 7 Bar chart showing the effects of Bent-Pro on seed germination under salt stress. *A. thaliana* seeds were sown on MS medium supplemented with 150 mM NaCl (black bar) or 150 mM NaCl combined with various concentrations of Bent-Pro (dark gray bars), Pro (light gray bars) or Bent (white bars). Germination was scored after 48 h. The percentage of germination with respect to the control for each treatment is shown. 100% indicates that the seeds germinated under control conditions (without NaCl). The data are mean values (±SE) of four independent experiments. ($n = 800$; t-Test * $p \leq 0.05$ **$p \leq 0.01$)

Because Bent addition showed no biological effects on seed germination under salinity, the action of Bent-Pro is probably associated with the bioactive effect of Pro within the Bent nanoclay. Plants are highly sensitive to Pro application doses, which is why optimizing the concentrations and time of application according to the crop that is to be treated and studied is essential (El Moukhtari et al. 2020). A similar result was reported by Deivanai et al. (2011) for the optimal concentration of exogenously applied Pro to alleviate the effect of NaCl on rice-seed germination.

To analyze further the putative protection effect of Bent-Pro against salinity in photosynthetic organs, the levels of chlorophyll were measured in 10-day-old *A. thaliana* seedlings treated with NaCl for 48 h in a hydroponic system (method of Iglesias et al. 2010, 2014). Salinity led to the loss of 50% of the chlorophyll content in leaves from plants subjected to NaCl-mediated stress for 48 h (Fig. 8). Leaves from plants exposed to salinity in combination with Bent-Pro showed only a very slight decrease in chlorophyll content, however, thus providing evidence of a protection against salt stress. When analyzing chlorophyll levels, Bent-Pro performed better in terms of the protection of *A. thaliana* plants from chlorosis symptoms than did free Pro, in accord with the germination results, suggesting emerging properties of the proposed bioactive nanocomposite. These results could be associated with a release of Pro from Bent-Pro for a more prolonged time period favoring plant uptake and a spatiotemporal induction of Pro-mediated signaling mechanisms (Jafarbeglou et al. 2016).

In agreement with the findings of the current study, Nanjo et al. (2003) reported that *A. thaliana* transgenic plants suppress Pro degradation and consequently accumulate higher levels of Pro and show reduced symptoms of salt stress in terms of chlorophyll levels. The contribution of Pro to salt-stress tolerance using transgenic plants has been the approach of agro-biotechnology investigations for many years (Per et al. 2017). Transgenic tobacco, sorghum, pigeon-pea, and citrumelo plants

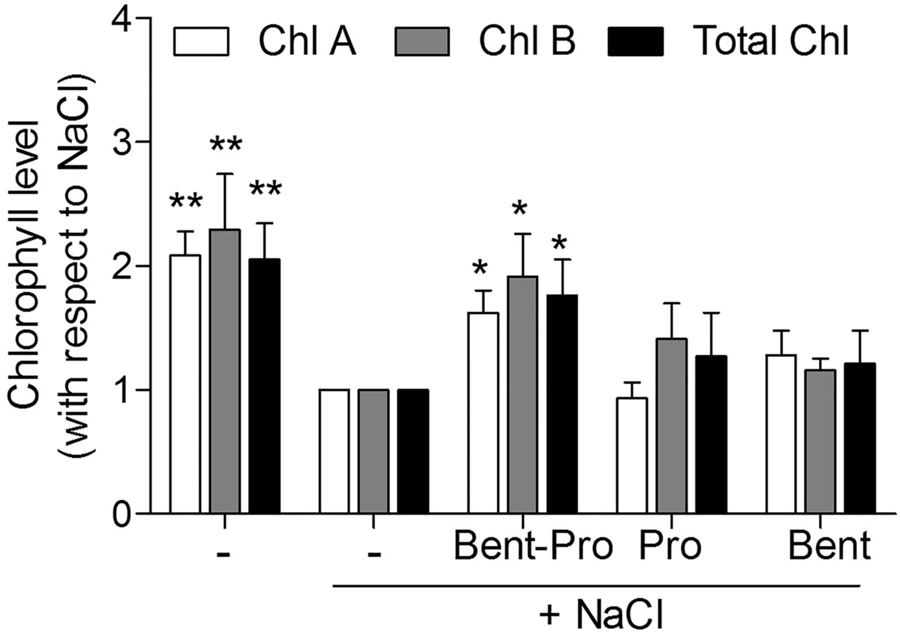

Fig. 8 Effects of Bent-Pro on seedlings grown under salt. 10-day-old *A. thaliana* seedlings grown in MS agar medium were transferred to liquid MS medium (control, -) or medium supplemented with 200 mM NaCl (+NaCl) in combination with 0.1 mg/mL Bent-Pro, 0.01 mg/mL Pro, and 0.1 mg/mL Bent for 48 h. The chlorophyll content was measured spectrophotometrically and related to control NaCl-treated seedlings. The data are mean values (±SE) of three independent experiments, ($n=240$; t-Test * $p \leq 0.05$ **$p \leq 0.01$)

that synthesize enhanced levels of Pro also presented an increased tolerance to salinity (de Campos et al. 2011; Kishor et al. 1995; Surekha et al. 2014; Surender Reddy et al. 2015). The potential effects on the environment and human health of transgenic biotechnology have led to questions amongst members of the public, however (Lu 2016). An alternative and quicker approach for the improvement of salt-stress tolerance indicates that the exogenous application of Pro exerts osmoprotection and favors crop productivity (Per et al. 2017), but the use of Bent-Pro offers a new approach to improving plant protection even more against salinity.

CONCLUSIONS

Biological tests indicated that Bent-Pro protected *A. thaliana* plants from NaCl-mediated inhibition of seed germination and the loss of chlorophyll, two typical physiological and biochemical markers of salt-mediated detrimental effects on plants. The method for obtaining optimal Bent-Pro nanoclay was described. Using twice the CEC of Bent as the initial amount of Pro led to ~ 14 wt.% of Pro being retained in the Bent and the reaction time influenced the intercalation reaction, with maximum retention of Pro being achieved after 2 h of reaction. Based on FTIR and TGA tests (and supported by SEM), Pro was present mainly in its zwitterionic form intercalated in the Bent interlayer space and on the edges or surfaces of its plates. The XRD and TEM analysis, together with the molecular-size calculations, confirmed intercalation of the Pro in Bent and indicated a monolayer arrangement of the Pro molecules in the Bent interlayer space. The results obtained indicated that the reaction mechanism consisted predominantly of the incorporation of Pro as a zwitterion due to its electrostatic interaction with Bent and the interlaminar cations. Bent-Pro thus constitutes an innovative bioactive nanocomposite that can improve the protection of plants against salinity.

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Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

Alexandre, M., & Dubois, P. (2000). Polymer-layered silicate nanocomposites: preparation, properties and uses of a new class of materials. *Materials Science and Engineering: R: Reports, 28*, 1–63.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. *Polyphenoloxidase in Beta vulgaris. Plant Physiology, 24*, 1–15.

Ashraf, M., & Foolad, M. R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany, 59*, 206–216.

Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil, 39*, 205–207.

Boyes, D. C., Zayed, A. M., Ascenzi, R., McCaskill, A. J., Hoffman, N. E., Davis, K. R., & Görlach, J. (2001). Growth stage–based phenotypic analysis of Arabidopsis. *The Plant Cell*, *13*, 1499LP–1510.

Bragg, W. H., & Bragg, W. L. (1913). The reflection of X-rays by crystals. *Proceedings of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 88*, 428–438.

Celis, R., Hermosín, M. C., Carrizosa, M. J., & Cornejo, J. (2002). Inorganic and organic clays as carriers for controlled release of the herbicide hexazinone. *Journal of Agricultural and Food Chemistry, 50*, 2324–2330.

Chen, H., Chen, Q. S., Huang, B., Wang, S. W., & Wang, L. Y. (2018). High-potential use of l-Cysh modified bentonite for efficient removal of U(VI). from aqueous solution. *Journal of Radioanalytical and Nuclear Chemistry, 316*, 71–80.

de Campos, M. K. F., de Carvalho, K., de Souza, F. S., Marur, C. J., Pereira, L. F. P., Filho, J. C. B., & Vieira, L. G. E. (2011). Drought tolerance and antioxidant enzymatic activity in transgenic ‘Swingle’ citrumelo plants over-accumulating proline. *Environmental and Experimental Botany, 72*, 242–250.

Deivanai, S., Xavier, R., Vinod, V., Timalata, K., & Lim, O. F. (2011). Role of exogenous proline in ameliorating salt stress at early stage in two rice cultivars. *Journal of Stress Physiology & Biochemistry, 7*, 157–174.

Devi, T. U., Lawrence, N., Ramesh Babu, R., Selvanayagam, S., Stoeckli-Evans, H., & Ramamurthi, K. (2009). Synthesis, crystal growth and characterization of l -Proline lithium chloride monohydrate: A new semiorganic nonlinear optical material. *Crystal Growth & Design, 9*, 1370–1374.

El Moukhtari, A., Cabassa-Hourton, C., Farissi, M., & Savouré, A. (2020). How does proline treatment promote salt stress tolerance during crop plant development? *Frontiers in Plant Science, 11*, 1127.

Food and Agriculture Organization of the United Nations. (2017). The future of food and agriculture: Trends and challenges. http://www.fao.org/3/I8429EN/i8429en.pdf

Gamba, M., Flores, F. M., Madejová, J., & Torres Sánchez, R. M. (2015). Comparison of imazalil removal onto montmorillonite and nanomontmorillonite and adsorption surface sites involved: an approach for agricultural wastewater treatment. *Industrial & Engineering Chemistry Research, 54*, 1529–1538.

Golldack, D., Li, C., Mohan, H., & Probst, N. (2014). Tolerance to drought and salt stress in plants: unraveling the signaling networks. *Frontiers in Plant Science, 5*, 151.

Grillo, R., Mattos, B. D., Antunes, D. R., Forini, M. M. L., Monikh, F. A., & Rojas, O. J. (2021). Foliage adhesion and interactions with particulate delivery systems for plant nanobionics and intelligent agriculture. *Nano Today, 37*, 101078.

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., & Ahmad, A. (2012). Role of proline under changing environments: a review. *Plant Signaling & Behavior, 7*, 1456–1466.

Iglesias, M. J., Terrile, M. C., Bartoli, C. G., D’Ippólito, S., & Casalongué, C. A. (2010). Auxin signaling participates in the adaptative response against oxidative stress and salinity by interacting with redox metabolism in Arabidopsis. *Plant Molecular Biology, 74*, 215–222.

Iglesias, M. J., Terrile, M. C., Windels, D., Lombardo, M. C., Bartoli, C. G., Vazquez, F., Estelle, M., & Casalongué, C. A. (2014). MiR393 regulation of auxin signaling and redox-related components during acclimation to salinity in Arabidopsis. *PLoS ONE, 9*, e107678.

Jafarbeglou, M., Abdouss, M., Shoushtari, A. M., & Jafarbeglou, M. (2016). Clay nanocomposites as engineered drug delivery systems. *RSC Advances, 6*, 50002–50016.

Kaur, G., & Asthir, B. (2015). Proline: a key player in plant abiotic stress tolerance. *Biologia Plantarum, 59*, 609–619.

Kishor, P. B. K., Hong, Z., Miao, G. H., Hu, C. A. A., & Verma, D. P. S. (1995). Overexpression of δ-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology*, *108*, 1387LP–1394.

Komadel, P., & Madejová, J. (2006). Acid activation of clay minerals. In Handbook of Clay Science (F. Bergaya, B.K.G. Theng, and G. Lagaly, (Ed.), *Developments in Clay Science.* (Vol. 1, pp. 263–287). Elsevier.

Lagaly, G. (2001). Pesticide–clay interactions and formulations. *Applied Clay Science, 18*, 205–209.

Li, J., Li, Y., & Dong, H. (2008). Controlled Release of Herbicide Acetochlor from Clay/Carboxylmethylcellulose Gel Formulations. *Journal of Agricultural and Food Chemistry, 56*, 1336–1342.

Lu, B.-R. (2016). Challenges of transgenic crop commercialization in China. *Nature Plants, 2*, 16077.

Mallakpour, S., & Dinari, M. (2011). Preparation and characterization of new organoclays using natural amino acids and cloisite Na^+. *Applied Clay Science, 51*, 353–359.

Mansilla, A. Y., Salcedo, M. F., Colman, S. L., Chevalier, M. T., Lanfranconi, M. R., Alvarez, V. A., & Casalongué, C. A. (2020). Characterization of functionalized bentonite as nanocarrier of salicylic acid with protective action against Pseudomonas syringae in tomato plants. *European Journal of Plant Pathology, 158*, 211–222.

McConnell, D. (1950). The crystal chemistry of montmorillonite. *American Mineralogist, 35*, 166–172.

McKee, T., & McKee, J. (2003). *Biochemistry: The Molecular Basis of Life*. McGraw-Hill.

Merino, D., & Alvarez, V. A. (2020). Thermal degradation of poly (ε-caprolactone). nanocomposites with soy lecithin-modified bentonite fillers. *Thermochimica Acta*, *689*, 178638.

Merino, D., Ollier, R., Lanfranconi, M., & Alvarez, V. (2016). Preparation and characterization of soy lecithin-modified bentonites. *Applied Clay Science, 127*, 17–22.

Merino, D., Mansilla, A. Y., Casalongué, C. A., & Alvarez, V. A. (2018). Preparation, characterization, and in vitro testing of nanoclay antimicrobial activities and elicitor capacity. *Journal of Agricultural and Food Chemistry, 66*(12), 3101–3109.

Merino, D., Tomadoni, B., Salcedo, M. F., Mansilla, A. Y., Casalongué, C. A., & Alvarez, V. A. (2020). Nanoclay as carriers of bioactive molecules applied to agriculture. Pp. 1–22 in: *Handbook of Nanomaterials and Nanocomposites for Energy and Environmental Applications* (O.V. Kharissova, L.M.T. Martínez, and B.I. Kharisov). Springer, Berlin.

Nanjo, T., Fujita, M., Seki, M., Kato, T., Tabata, S., & Shinozaki, K. (2003). Toxicity of free proline revealed in an arabidopsis T-DNA-tagged mutant deficient in proline dehydrogenase. *Plant & Cell Physiology, 44*, 541–548.

Negrão, S., Schmöckel, S. M., & Tester, M. (2016). Evaluating physiological responses of plants to salinity stress. *Annals of Botany, 119*, 1–11.

Ni, B., Liu, M., Lü, S., Xie, L., & Wang, Y. (2011). Environmentally Friendly Slow-Release Nitrogen Fertilizer. *Journal of Agricultural and Food Chemistry, 59*, 10169–10175.

Parbhakar, A., Cuadros, J., Sephton, M. A., Dubbin, W., Coles, B. J., & Weiss, D. (2007). Adsorption of l-lysine on montmorillonite. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 307*, 142–149.

Patel, H. A., Somani, R. S., Bajaj, H. C., & Jasra, R. V. (2006). Nanoclays for polymer nanocomposites, paints, inks, greases and cosmetics formulations, drug delivery vehicle and waste water treatment. *Bulletin of Materials Science, 29*, 133–145.

Pédro, G. (1965). La constitution cristallochimique des phyllosilicates. Sa signification et ses conséquences. *Bulletin du Groupe français des argiles, 16*, 37–47.

Per, T. S., Khan, N. A., Reddy, P. S., Masood, A., Hasanuzzaman, M., Khan, M. I. R., & Anjum, N. A. (2017). Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. *Plant Physiology and Biochemistry, 115*, 126–140.

Petra, L., Billik, P., & Komadel, P. (2015). Preparation and characterization of hybrid materials consisting of high-energy ground montmorillonite and α-amino acids. *Applied Clay Science, 115*, 174–178.

Ruiz-Hitzky, E., Aranda, P., Darder, M., & Rytwo, G. (2010). Hybrid materials based on clays for environmental and biomedical applications. *Journal of Materials Chemistry, 20*, 9306–9321.

Saha, J., Brauer, E. K., Sengupta, A., Popescu, S. C., Gupta, K., & Gupta, B. (2015). Polyamines as redox homeostasis regulators during salt stress in plants. *Frontiers in Environmental Science, 3*, 21.

Sanchez-Martin, M. J., Rodriguez-Cruz, M. S., Andrades, M. S., & Sanchez-Camazano, M. (2006). Efficiency of different clay minerals modified with a cationic surfactant in the adsorption of pesticides: influence of clay type and pesticide hydrophobicity. *Applied Clay Science, 31*, 216–228.

Shahid, S. A., Zaman, M., & Heng, L. (2018). Introduction to soil salinity, sodicity and diagnostics techniques. Pp. 1–42 in: *Guideline for Salinity Assessment*, *Mitigation and Adaptation Using Nuclear and Related Techniques* (M. Zaman, S.A. Shahid, and L. Heng, editors). Springer International Publishing, Cham.

Shokri, E., Yegani, R., & Akbarzadeh, A. (2017). Novel adsorptive mixed matrix membranes by embedding modified montmorillonite with arginine amino acid into polysulfones for As(V) removal. *Applied Clay Science, 144*, 141–149.

Surekha, C., Kumari, K. N., Aruna, L. V., Suneetha, G., Arundhati, A., & Kavi Kishor, P. B. (2014). Expression of the *Vigna aconitifolia* P5CSF129A gene in transgenic pigeonpea enhances proline accumulation and salt tolerance. *Plant Cell. Tissue and Organ Culture (PCTOC), 116*, 27–36.

Surender Reddy, P., Jogeswar, G., Rasineni, G. K., Maheswari, M., Reddy, A. R., Varshney, R. K., & Kavi Kishor, P. B. (2015). Proline over-accumulation alleviates salt stress and protects photosynthetic and antioxidant enzyme activities in transgenic sorghum [Sorghum bicolor (L.). Moench]. *Plant Physiology and Biochemistry, 94*, 104–113.

Szabados, L., & Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science, 15*, 89–97.

Wu, C.-J., Gaharwar, A. K., Schexnailder, P. J., & Schmidt, G. (2010). Development of biomedical polymer-silicate nanocomposites: A materials science perspective. *Materials, 3*, 2986–3005.

Zhang, J. Z., Creelman, R. A., & Zhu, J.-K. (2004). From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiology*, *135*, 615LP–621.

Zhu, J.-K. (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology, 53*, 247–273.

Zhu, J., Zhang, P., Qing, Y., Wen, K., Su, X., Ma, L., Wei, J., Liu, H., He, H., & Xi, Y. (2017). Novel intercalation mechanism of zwitterionic surfactant modified montmorillonites. *Applied Clay Science, 141*, 265–271.

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