An Evaluation of Maleic-Itaconic Copolymers as Urease Inhibitors

Urea hydrolysis in soil is accelerated 10\(^{15}\)-fold by the nickel-dependent enzyme urease to yield a source of N that can be assimilated by plants. This reaction determines an overall soil pH increase and significant ammonia volatilization, decreasing the efficiency of urea-based fertilization. A control of urease activity is thus required for agronomic purposes. Maleic-Itaconic Polymers (MIPs) have been claimed to decrease the N loss as ammonia volatilization by inhibiting urease activity. To investigate this matter, we performed an in vitro study using urease from jack bean (Canavalia ensiformis, JBU) at pH 7.5 and pH 5.0. Urease is not affected by MIPs at pH 7.5, but the enzyme is completely inactivated in 20 min at pH 5.0 using MIPs in the 0.4 to 2.4 \(\mu\text{mol L}^{-1}\) range. This inactivation is comparable to that attained using similar concentrations of \(N-(n\text{-butyl})\text{-thiophosphoric triamide (NBPT).}\)

In vivo assays conducted using Sporosarcina pasteurii as a model for a widespread soil bacterium and urea, in the presence of either MIPs or NBPT at pH 7.5, revealed that NBPT significantly inhibits both cellular growth and urease activity, while MIPs have no effect. To elucidate the mechanism of extracellular urease inactivation by MIPs in vitro, their Ni(II) sequestration capability was investigated. The MIPs (50 \(\mu\text{mol L}^{-1}\)) completely extract the essential Ni(II) ions from the active site of urease at pH 5.0, in 3 h. This phenomenon is related to the capability of MIPs to shift the Ni(II)-urease dissociation equilibrium through binding and chelation of the Ni(II) ions off the active site.

Abbreviations: EDTA, ethylene-diamine-tetraacetic acid; JBU, urease from jack bean; MIP, maleic-itaconic polymer; NBPT, \(N-(n\text{-butyl})\text{-thiophosphoric triamide.}\)

Core Ideas

- Maleic-Itaconic Polymers (MIPs) strongly and rapidly inactivate urease at pH 5.0.
- MIPs favour the release of the essential Ni(II) ions from the active site of urease.
- The urease inactivation by MIPs is comparable with that attained using NBPT.

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Urea is currently the most popular N fertilizer source, accounting for more than 60% of the world market (Prud’homme, 2016). According to the International Fertilizer Association, 183 million metric tons of urea were produced in 2017, representing the major sectorial growth in the N industry in terms of demand and production (Prud’homme, 2016). This growth is the direct consequence of urea’s lower price, higher N content, and reduced transport and distribution costs as compared to ammonium nitrate. In soil, urea undergoes fast enzymatic hydrolysis to yield inorganic N sources for plant nutrition through the activity of the enzyme urease (urea aminohydrolase, EC 3.5.1.5), by means of the reaction shown in Fig. 1.

Urease is a nickel-dependent, non-redox enzyme that accelerates urea hydrolysis to a rate 10\(^{15}\) times higher than the non-catalyzed reaction, making it the most efficient enzyme known to date (Zambelli et al., 2011; Maroney and Ciurli, 2014; Mazzei et al., 2017b). However, the use of urea as a N fertilizer causes a number of significant agronomic, environmental and economic problems (Englestad and Hauck, 1974; Beaton, 1978; Kiss and Simihaian, 2002). Indeed, it has been suggested that only 47% of the applied urea N is taken up by crops (Galloway and Cowling, 2002). The N loss from urea is due in part to the rapid
active site cavity of ureases is largely conserved, with the enzyme using x-ray diffraction crystallography (Maroney and Ciurli, 2014; Mazzei et al., 2017b). These structures revealed that the enzyme appears to limit influx as well as the subsequent assimilation pathway, negatively affecting plant growth.

Zea mays maize (L.) plants (Zanin et al., 2015, 2016). The exposure of NBPT interferes with urea nutrition in hydroponically grown plants (Bremner and Mulvaney, 1978; Chien et al., 2009). Several classes of molecules have been proposed and tested as urease inhibitors (Krajewska et al., 2001; Zaborska et al., 2004; Krajewska, 2009; Kosikowska and Berlicki, 2011). Among the thousands of compounds or mixtures thereof, evaluated for their effect on soil urease (Kiss and Simihaiarn, 2002), the most widely utilized urease inhibitor in agronomic settings is NBPT, whose effectiveness has been characterized in a number of studies, both in vitro and in soils (Bremner and Chai, 1986; McCarty et al., 1989; Christianson et al., 1990; Creason et al., 1990; Bremner et al., 1991; Hendrickson and Douglass, 1993; Keerthsinghe and Blakeley, 1995). Recently, the kinetic and structural details of urease inhibition by NBPT have been elucidated at the molecular level (Mazzei et al., 2017a). However, NBPT was shown to have some negative aspects (Bremner, 1995), with noticeable indications of NBPT toxicity, such as leaf necrosis, in sorghum (Sorghum bicolor L.), wheat (Triticum aestivum L.), rye (Secale cereale L.), and pea (Pisum sativum L.) (Krogmeier et al., 1989; Watson and Miller, 1996; Artola et al., 2011; Cruchaga et al., 2011). The toxicity of NBPT for plants can be explained by considering an elevated urea concentration within the plant tissue stemming from reduced endogenous urease activity. Recently, it has been observed that the presence of NBPT interferes with urea nutrition in hydroponically grown maize (Zea mays L.) plants (Zanin et al., 2015, 2016). The presence of NBPT in the urea-containing nutrient solution appears to limit influx as well as the subsequent assimilation pathway, negatively affecting plant growth.

In an effort to design and develop more efficient urease inhibitors to improve the efficiency of soil fertilization and crop production, high-resolution structures of ureases from bacterial and plant sources have been established in the last two decades using x-ray diffraction crystallography (Maroney and Ciurlči, 2014; Mazzei et al., 2017b). These structures revealed that the active site cavity of ureases is largely conserved, with the enzyme activity relying on the presence of two Ni(II) ions coordinated to a carboxylate side chain of aspartate, to a carbamylated lysine, as well as to imidazole groups of histidines (Fig. 2).

Knowledge of the structures of urease-inhibitor complexes has thus been exploited to identify a long list of molecules that act as urease inhibitors (Krajewska, 2009; Kosikowska and Berlicki, 2011; Maroney and Ciurlči, 2014; Mazzei et al., 2017b). In particular, the inhibitors for which both kinetic and structural aspects have been established can be roughly divided into two main classes. One class is composed of substances such as β-mercapto-ethanol (Benini et al., 1998), 1,4-benzoquinone (Mazzei et al., 2016b) and catechol (Mazzei et al., 2017c). These molecules have been proven to target the Sγ atom of a conserved cysteine residue located onto a mobile helix-turn-helix motif (also called mobile flap) in the vicinity of the active site. This cysteine residue is essential for catalysis by modulating the transit of substrate and products through the active site cavity. The second class of urease inhibitors contains substances such as phosphoramidates (Benini et al., 1999; Mazzei et al., 2017a), phosphate (Benini et al., 2001), thiols (Benini et al., 1998), sulfite (Mazzei et al., 2016a), fluoride (Benini et al., 2014), hydroxamic (Benini et al., 2000), boric (Benini et al., 2004) and citric (Benini et al., 2013) acids. These molecules inhibit urease by directly binding to the Ni(II) ions in the active site. β-mercapto-ethanol can actually be ascribed to both classes, because it is able to use its thiol moiety to bridge the Ni(II) ions in the active site as well as to covalently modify the essential and conserved cysteine residue on the mobile flap (Benini et al., 1998).

Citrate is a noteworthy case: as a tri-carboxylic acid, this molecule was shown to act as a urease inhibitor by directly coordinating the Ni(II) ions in the urease active site using one of its three carboxylate moieties (Benini et al., 2013). This observation is relevant to recent reports indicating that poly-carboxylic acid poly-
mers, made of maleic and itaconic acids (maleic-itaconic polymers, or MIPs [Sanders et al., 2003, 2004]) and sold as Nutrisphere-N (Verdesian Life Sciences LLC, Cary, NC) could act as urease inhibitors. It has been indeed shown that MIPs can reduce ammonia volatilization and improve the agronomic efficiency of urea-based N fertilizers, by reducing N loss and the associated yield loss that accompanies insufficient N availability as compared to the same products without the copolymer treatment (Blaylock and Murphy, 2006; Heininger, 2011; Gordon, 2013; Mooso and Tindall, 2013; Dunn and Wiatrak, 2014; Heininger et al., 2014; Wiatrak, 2014; Wiatrak and Gordon, 2014). In particular, MIPs have been reported to inactivate soil urease by extracting the Ni(II) ions from the active site of the enzyme (Blaylock and Murphy, 2006), but no studies confirming this hypothesis have been reported in the literature. These claims contrast with other field and laboratory studies that concluded that Nutrisphere-N has no urea volatilization inhibiting properties at the recommended rates (Franzen et al., 2011; Goos, 2013a). Some reduction of ammonia loss through the use of this product has been reported (Goos, 2013b; Wiatrak and Gordon, 2014), with the observed effect suggested to depend on the decrease of soil pH by the highly acidic poly-carboxylate polymer (Goos, 2013b). Indeed, the use of urea fertilizer coated with Nutrisphere-N has been reported to significantly decrease the soil surface pH in both dry and wet conditions, an observation that was correlated to a decrease in ammonia loss and nitrate leaching (Peng et al., 2015).

The latter hypothesis stimulated the undertaking of the present study, which is a re-evaluation of MIPs as urease inactivators performed by comparing their enzymatic reduction efficiency at pH 7.5 and pH 5.0. In fact, it is known that urease activity depends on pH, with an optimum around pH 7.5 and a bell-shaped profile covering the 5 to 9 pH range (Giurliti et al., 1996; Kajewska, 2009). The loss of urease activity at pH ≤ 5 can be generally ascribed to protonation events occurring at the level of the Ni(II)-coordinating residues in the enzyme active site (Fig. 2), which cause the loss of the metal ions and the consequent irreversible inactivation of the enzyme. This inactivation mechanism was proven to occur for urease from jack bean (Canavalia ensiformis, JBU) by using ethylene-diamine-tetraacetic acid (EDTA) as a chelating agent at low pH (Dixon et al., 1980). This process cannot be reverted by simple addition of Ni(II) to the apo-urease in vitro, because Ni incorporation in the apo-enzyme occurs through a complex in vivo maturation process that requires the action of several accessory proteins and a metabolically active cell (Zambelli et al., 2011). The objective of the present study is thus the assessment of the capability of MIPs to inactivate urease, as a function of pH. A comparison of MIPs and NBPT as urease inhibitors at two different pH values is also presented.

**MATERIALS AND METHODS**

**Materials**

The maleic-itaconic copolymers BC (2-methylene-butenedioic acid, polymer with 2,5-furandione, hydrogen peroxide-initiated, hydrolyzed, CAS 1613322-33-0) and T5 (2-methylenebutenedioic acid, polymer with 2,5-furandione, sulfonic acid, 2-methyl-2-propene, and sulfonic acid, 2-propene, hydrogen peroxide-initiated, hydrolyzed, partial sodium salt, CAS 2197089-06-6) were provided by Verdesian Life Sciences LLC. The polymers are water solutions at 40% w/v, according to the manufacturer's indications, and were used according to this labeling. The number-averaged molecular weight, provided by the manufacturer for both BC and T5, is approximately 2.75 ± 0.25 kDa, with a weight-averaged molecular weight in 2.8 to 4.0 kDa range, determined by Ultrahydrogel 120 and 250 size exclusion chromatography columns (Waters Co., Milford, MA) at 35°C, using aqueous 0.1 mol L\(^{-1}\) sodium nitrate as eluent and poly(ethylene glycol) and poly(ethylene oxide) as standards. Urease from jack bean (Canavalia ensiformis, JBU) C3-type was purchased from Sigma-Aldrich (Saint Louis, MO) and quantified following the manufacturer's instructions. \(N\)-(n-butyl)-thiophosphoric triamide (NBPT, CAS 94317-64-3) was purchased from Apollo Scientific (Bredbury, UK). Ethylene-diamine-tetraacetate sodium salt (EDTA, CAS 60-00-4) was acquired from Giotto Biotech (Sesto Fiorentino, Italy). Maleic acid (CAS 110-16-7), and itaconic acid (CAS 97-65-4) were purchased from Sigma Aldrich (Saint Louis, MO). *Sporosarcina pasteurii* (DSMZ 33) was purchased from DSMZ (Braunschweig, Germany).

**Enzymatic Assay Using the pH-STAT Method**

Urease activity was determined in triplicate using the pH-STAT method, as described by (Blakeley et al., 1969). In particular, a T1 pH-meter equipped with a 50-14 T electrode (Crisson Instruments, SA), was used to record, every 0.5 min, the volume of a 50 mmol L\(^{-1}\) HCl solution necessary to maintain the pH of a solution containing urease at a determined and constant value. The measurement started 0.5 min after urea addition to allow time to reach uniform substrate concentration in the sample volume. One unit of enzyme is defined as the amount of urease required to hydrolyze 1 \(\mu\)mol urea min\(^{-1}\) of reaction.

The reaction mixture (10 mL) was composed of 9.7 mL of 2 mmol L\(^{-1}\) 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer at pH 7.5 or 2 mmol L\(^{-1}\) 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 5.0, containing one of the following:

(i) 1, 3, and 6 mg L\(^{-1}\) (0.4, 1.2 and 2.4 \(\mu\)mol L\(^{-1}\)) of BC;
(ii) 1, 3, and 6 mg L\(^{-1}\) (0.4, 1.2 and 2.4 \(\mu\)mol L\(^{-1}\)) of T5;
(iii) 10 mmol L\(^{-1}\) EDTA;
(iv) 1, 3, and 6 mg L\(^{-1}\) (6, 18, and 36 \(\mu\)mol L\(^{-1}\)) NBPT
(v) 6 and 60 mg L\(^{-1}\) (52 and 520 \(\mu\)mol L\(^{-1}\)) maleic acid at pH 5.0; and
(vi) 6 and 60 mg L\(^{-1}\) (46 and 460 \(\mu\)mol L\(^{-1}\)) itaconic acid at pH 5.0.

Stock solutions of MIPs were adjusted to pH 7.5 or 5.0 by addition of NaOH prior to addition to the corresponding buffer. A urease solution (0.2 mL) in each corresponding buffer was added
to the reaction mixture to a final concentration of 5 nmol L\(^{-1}\). Independent samples were incubated for different periods (0, 5, 10, and 20 min) before starting the measurement of urease activity. The latter consisted in the addition of 0.1 mL of a concentrated solution of urea dissolved in the same buffer to the urease solutions described above, to reach a final concentration of 100 mM, and continued for 3 min under constant magnetic stirring.

A two-way analysis of variance (ANOVA) test with Bonferroni correction was used to analyze the statistical significance (expressed as a p-value) between the samples and the corresponding references in terms of inhibitor concentration \((p_{\text{conc}})\), incubation time \((p_{\text{time}})\), and their interaction \((p_{\text{int}})\). In the case of the experiments performed in the presence of EDTA, the statistical analysis was performed by one-way ANOVA and Tukey exploited as a post-hoc test. A p-value ≤ 0.05 was chosen as a threshold value to establish whether a statistical significance was present or not. Results were finally expressed as mean ± standard error (SE) of the mean. All graphics and statistical analysis were done using GraphPad Prism 5.0 (San Diego, CA).

### In-vivo Enzymatic Assay Using the UV-Vis Method

Urease activity was spectrophotometrically determined in triplicate by means of a modified version of the protocol described by (Mazzei et al., 2016b), using cresol red as a probe to follow the increase of pH over time, due to urease activity. An overnight *Sporosarcina pasteurii* (DSM 33) culture, grown at 30°C and constant stirring in a medium containing 10 g L\(^{-1}\) of yeast extract and 150 mmol L\(^{-1}\) urea, both dissolved in 130 mmol L\(^{-1}\) HEPES buffer at pH 8.0, was split in independent aliquots being inoculated in the same medium, obtaining a starting optical density at 600 nm \((\text{OD}_{600})\) of 0.2. Each sample was added with either 2 mmol L\(^{-1}\) NBPT, 6 mg L\(^{-1}\) BC or 6 mg L\(^{-1}\) T5 polymers. A reference sample was grown in the absence of any of the testing molecules, and its cellular growth, as determined by the OD\(_{600}\) index, was taken as 100% reference. After appropriate periods of time, OD\(_{600}\) was independently measured for each sample. At the same time points, aliquots of 10 µL from each culture were added to 990 µL of a solution containing 30 mg L\(^{-1}\) cresol red and 100 mmol L\(^{-1}\) urea dissolved in 2 mmol L\(^{-1}\) HEPES buffer at pH 7.5 to determine the amount of urease activity. Measurements were performed at room temperature with a Cary 60 UV-Vis Spectrophotometer (Agilent, Milan, Italy). The change in absorbance over time, due to the change in color of cresol red, was monitored at λ = 573 nm. The activity was then calculated by a linear fitting of the straight portion in the absorbance vs. time curve and normalized to the residual activity value measured at time zero of incubation. Residual activity values were standardized to the optical density to minimize any effect due to different growth rates in the samples. The obtained results (expressed as percentage of the enzyme activity in the sample devoid of either MIPs or NBPT) were plotted as a function of time.

A two-way ANOVA test with Bonferroni correction was used to analyze the statistical significance between the samples and the corresponding references by using the same procedure described for the in vitro experiments. A p-value ≤ 0.05 was chosen as a threshold value to establish whether a statistical significance was present or not. Results were finally expressed as mean ± standard error (SE) of the mean. All graphics and statistical analysis were done using GraphPad Prism 5.0 (San Diego, CA).

### Determination of Ni Content in Urease in the Presence of MIPs

A stock solution (300 µL) of 2.5 µmol L\(^{-1}\) JBU in 5 mmol L\(^{-1}\) HEPES buffer at pH 7.0 was diluted to a final volume of 8 mL using 2 mmol L\(^{-1}\) MES buffer, at pH 5.0, in the absence or in the presence of 120 mg L\(^{-1}\) of BC or T5 (50 µmol L\(^{-1}\)). Analogous experiments were performed using 10 mmol L\(^{-1}\) EDTA instead of BC or T5 polymers. After 3 h of incubation, each solution was filtered using a 10 kDa molecular weight cutoff Centricron (Darmstadt, Millipore) membrane, collecting identical volumes (4 mL) of the retained and the flow-through solutions. The chosen molecular weight cutoff allows for urease (Mr approximately 550 kDa) to be retained by the semi-permeable membrane, while the MIPs are able to pass through. The concentration of polymers was dictated by the amount of urease necessary for Ni to be detectable by inductively coupled plasma–optical emission spectrometry (ICP–OES) and chosen to fall within the range of the MIPs/urease ratios used in the pH-STAT experiments. The Ni content of each portion (retained and flow-through) was measured using an ICP–OES Spectro Arcos instrument (SPECTRO Analytical Instruments GmbH, Kleve, Germany), as described by (Stola et al., 2006) and the respective amount of Ni was normalized to the total Ni amount as a percentage. Each experiment was performed in triplicate. The content of Ni in MIPs or EDTA was determined to be null using the same protocol. One-way ANOVA and Tukey tests were used to analyze the statistical significance between each sample and the corresponding reference. A p-value ≤ 0.05 was chosen as a threshold value to establish whether a statistical significance was present or not. The results were expressed as mean ± standard error (SE) of the mean. All graphics and statistical analysis were done using GraphPad Prism 5.0 (San Diego, CA).

### RESULTS AND DISCUSSION

#### In-vitro Enzymatic Assays

The effects of BC and T5 polymers on the activity of urease were evaluated at pH 7.5 and at pH 5.0, and the corresponding results are shown in Fig. 3A and B, respectively. A comparison with EDTA, NBPT, and with the monomeric fragments of MIPs (maleic and itaconic acids) was also performed in the same experimental conditions using a consistent methodology for the measurement of enzymatic activity. The BC and T5 do not affect urease activity at pH 7.5 at concentrations in the range 0.3 to 1.8 µmol L\(^{-1}\) (Fig. 3A and B). At the same pH value, 10 mmol L\(^{-1}\) EDTA does not inhibit urease (Fig. 3C). On the other hand, NBPT strongly inhibits urease at 7.5 pH, at concentrations similar to those used for BC and T5 (Fig. 3D). This is an important observation, as the amount of NBPT or MIPs commercially applied to fertilizer is very similar (Goos, 2013a). The ANOVA
shows that NBPT inhibits urease significantly at pH 7.5 (Fig. 3D), for NBPT concentration, incubation time and an interaction between them are relevant for the enzyme inhibition ($p_{\text{conc}}$, $p_{\text{time}}$ and $p_{\text{int}} \leq 0.0001$). The inhibition of urease activity among BC, T5, EDTA and NBPT was not statistically compared, but numerically, the percentage of urease activity in the presence of NBPT is strongly decreased, while no significant effect is observed in the other three cases.

A totally different result is obtained at pH 5.0 (Fig. 4), a condition in which the inactivation of urease by BC ($p_{\text{conc}} \leq 0.0001$, $p_{\text{time}} = 0.004$, $p_{\text{int}} = 0.1341$) and T5 ($p_{\text{conc}} \leq 0.0001$, $p_{\text{time}} = 0.0252$, $p_{\text{int}} = 0.0766$) in the μmol L$^{-1}$ concentration range is clearly observable, with a concentration-dependent inhibition profile that eventually results in a complete loss of urease activity (Fig. 4A and B). Under similar conditions, 10 mmol L$^{-1}$ EDTA inhibits urease in a time-dependent manner (Fig. 4C; $p \leq 0.0001$). This is consistent with previously reported studies (Dixon et al., 1980). In the case of EDTA, the inactivation of urease does not achieve a complete abolishment of enzymatic catalysis, as observed for BC and T5, in the range of concentrations used. The effect of either BC or T5 on urease activity at pH 5.0 (Fig. 4D) is comparable to that of NBPT ($p_{\text{conc}}$, $p_{\text{time}}$ and $p_{\text{int}} \leq 0.0001$). At pH 5.0, neither maleic (Fig. 4E) nor itaconic (Fig. 4F) acids show any urease inhibition properties, suggesting that the polymeric nature of MIPs induces the observed effect on urease activity.

**Evaluation of Urease Inhibition by MIPs and NBPT Using In-vivo Assays**

The ability of cells of the widespread soil bacterium *Sporosarcina pasteurii* to grow and to express an active urease enzyme in solution at pH 7.5, in the presence or in the absence of NBPT (Fig. 5) and MIPs (Fig. 6) was investigated using urea as N source. The same experiments were conducted using ammonium sulfate for comparison, as the utilization of the latter for cell growth does not require urease activity. In all cases, the experiments performed using urea in the absence of any inhibitor have been considered as references (Fig. 5 and 6). The results

Fig. 3. Residual percentage activity of jack bean urease (JBU), referred to 100% (REF, black bar) after incubation for different time periods at increasing concentrations of BC (2-methylene-butandioic acid, polymer with 2,5-furandione, hydrogen peroxide-initiated, hydrolyzed, CAS 1613322-33-0, Panel A), T5 (2-methylene-butandioic acid, polymer with 2,5-furandione, sulfonic acid, 2-methyl-2-propene, and sulfonic acid, 2-propene, hydrogen peroxide-initiated, hydrolyzed, partial sodium salt, CAS 2197089-06-6, Panel B), EDTA (ethylene-diamine-tetraacetic acid, Panel C), and NBPT (N-(α-butyl)-thiophosphoric triamide, Panel D), at pH 7.5. In Panels A and B, the red, orange and blue bars correspond to 1, 3, and 6 mg L$^{-1}$ (0.4, 1.2 and 2.4 μmol L$^{-1}$), respectively. In Panel C, the concentration of EDTA is 10 mmol L$^{-1}$. In Panel D, the same colors are used to indicate 1, 3, and 6 mg L$^{-1}$ (6, 18, and 36 μmol L$^{-1}$) of NBPT. Mean ± SE (n = 3). *** Significant at $p \leq 0.001$. 

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presented in Fig. 5A show that bacterial cell growth, using urea as the sole N source, is negatively and significantly affected ($p_{\text{conc}}, p_{\text{time}}$, and $p_{\text{int}} \leq 0.0001$) by the presence of 2 mmol L$^{-1}$ NBPT (Fig. 5A). When urea is replaced with ammonium sulfate as the N source, no significant difference in cell growth is observed as compared with the reference, either in the absence or in the presence of 2 mmol L$^{-1}$ NBPT (Fig. 5A).

![Graphs of residual percentage activity of jack bean urease (JBU), referred to 100% (REF, black bar), after incubation for different time periods at increasing concentrations of BC (2-methylene-butanedioic acid, polymer with 2,5-furandione, hydrogen peroxide-initiated, hydrolyzed, CAS 1613322-33-0, Panel A), T5 (2-methylene-butanedioic acid, polymer with 2,5-furandione, sulfonic acid, 2-methyl-2-propene, and sulfonic acid, 2-propene, hydrogen peroxide-initiated, hydrolyzed, partial sodium salt, CAS 2197089-06-6, Panel B), EDTA (ethylene-diamine-tetraacetic acid, Panel C), NBPT (N-(n-butyl)-thiophosphoric triamide, Panel D), maleic acid (Panel E), and itaconic acid (Panel F), at pH 5.0. In Panels A and B, the red, orange, and blue bars correspond to 1, 3, and 6 mg L$^{-1}$ (0.4, 1.2, and 2.4 mmol L$^{-1}$), respectively. In Panel C, the concentration of EDTA is 10 mmol L$^{-1}$. In Panel D, the same colors are used to indicate 1, 3, and 6 mg L$^{-1}$ (6, 18, and 36 µmol L$^{-1}$) of NBPT. In Panel E, the red and blue bars correspond to 6 and 60 mg L$^{-1}$ (52 and 520 µmol L$^{-1}$) maleic acid. In Panel F, the red and blue bars correspond to 6 and 60 mg L$^{-1}$ (46 and 460 µmol L$^{-1}$) itaconic acid. Mean ± SE. *** Significant at $p \leq 0.001$. 

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The results presented in Fig. 5B show the dependence of urease activity as a function of the N source, either urea or ammonium sulfate, in the absence or in the presence of NBPT. The results indicate that a significant decrease ($p_{\text{conc}}, p_{\text{time}}$ and $p_{\text{int}} \leq 0.0001$) of urease activity occurs when 2 mmol L$^{-1}$ NBPT is used in the cell culture (Fig. 5B). Consistent with the lack of requirement for urease activity in the case of cells grown in a medium containing an already available N source, the replacement of urea with ammonium sulfate results in a strong decrease of urease activity either in the absence or in the presence of NBPT.

To investigate the ability of S. pasteurii to grow and to express an active form of urease in the presence of MIPs, the experiments described for NBPT were repeated using these compounds in the presence of urea as a N source at pH 7.5 (Fig. 6). The profiles for cellular growth (Fig. 6A) and for urease activity (Fig. 6B) do not appear to be influenced by the presence of MIPs. These results corroborate the in vitro analysis described in the previous section (Fig. 3A and B), confirming that MIPs are able to inhibit urease activity at pH 5.0 but not at pH 7.5. Interestingly, MIPs do not appear to have any negative influence on the rate of cellular growth at pH 7.5, suggesting that they are not toxic for S. pasteurii cells in the experimental conditions used.

Nickel Sequestration Experiments

Having established a strong inactivation of urease by MIPs at pH 5.0, and to shed light on the mode of action for this effect, the capability of BC and T5 copolymers to sequestrate Ni from the bimetallic Ni(II) center of the enzyme active site was evaluated using ICP–OES measurements.

As shown in Fig. 7, when JBU was incubated without the polymers (negative control), approximately 80 and 20% of the total amount of Ni was detected in the retained solution and in the cell culture respectively. The results of these experiments are shown in Fig. 7A and B, where the percentage of Ni retained by the polymers is plotted as a function of time.

![Fig. 5.](image1)

**Fig. 5.** *Sporosarcina pasteurii* cellular growth (Panel A) and urease activity (Panel B) in the absence or in the presence of 2 mmol L$^{-1}$ NBPT (N-(n-butyl)thiophosphoric triamide). Cell growth is shown as a percentage value, being the cell optical density at 600 nm (OD$_{600}$) in the absence of either MIPs or NBPT taken as 100%. Urease activity was normalized to the number of cells and then shown as a percentage value. The color code used in both Panels A and B is the following: cellular growth (or urease activity) using urea as N source in the absence or in the presence of 2 mmol L$^{-1}$ NBPT is shown as red and in blue bars, respectively; cellular growth (or urease activity) using ammonium sulfate as a N source, in the absence or in the presence of 2 mmol L$^{-1}$ NBPT is shown as orange and in light blue bars, respectively. Mean ± SE (n = 4). *** Significant at $p \leq 0.001$.

![Fig. 6.](image2)

**Fig. 6.** *Sporosarcina pasteurii* cellular growth (Panel A) and urease activity (Panel B) in the absence or in the presence of 6 mg L$^{-1}$ of BC (2-methylene-butanedioic acid, polymer with 2,5-furandione, hydrogen peroxide-initiated, hydrolyzed, CAS 1613322-33-0) and T5 (2-methylene-butanedioic acid, polymer with 2,5-furandione, sulfonic acid, 2-methyl-2-propene, and sulfonic acid, 2-propene, hydrogen peroxide-initiated, hydrolyzed, partial sodium salt, CAS 2197089-06-6). Cell growth is shown as a percentage value, being the cell optical density at 600 nm (OD$_{600}$) in the absence of either MIPs or NBPT taken as 100%. Urease activity was normalized to the number of cells and then shown as a percentage value. The color code used in both Panels A and B is the following: cellular growth (or urease activity) using urea as nitrogen source in the absence, in the presence of 6 mg L$^{-1}$ T5, or 6 mg L$^{-1}$ BC are shown as red, blue, and light blue bars, respectively. Mean ± SE. *** Significant at $p \leq 0.001$. 

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the flow-through, respectively. On the other hand, when JBU was incubated with BC and T5 polymers, the amount of Ni detected in the retained solution decreased to approximately 55% of total Ni, while the flow-through Ni content increases to approximately 45% of total Ni. The increase of the Ni content in the flow-through indicates a significant loss of the Ni(II) ions from the active site of urease triggered by the presence of the MIPs ($p \leq 0.0001$). Indeed, in the hypothesis of a total removal of Ni from the active site of urease, both of these percentages would be 50% because the volumes of the retained and flow-through portions are identical. Therefore, approximately 90% of Ni is extracted from urease in these conditions, which is consistent with the effectively total inactivation of enzymatic activity as established using the pH-STAT method.

As a positive control, the same experiment performed in the presence of 10 mmol L$^{-1}$ EDTA gave a smaller, but appreciable, increase of Ni content in the flow-through, suggesting that the effect of EDTA as a chelating agent, facilitating extraction of Ni(II) from the urease active site, is smaller than the MIPs in the experimental conditions used.

The determination of JBU activity in the presence of two different MIPs formulations at pH 7.5 and pH 5.0 indicates that the MIPs have no significant inhibitory effect on urease activity at pH 7.5, a condition in which, on the other hand, NBPT inhibits the enzyme (Fig. 3). This result confirms previous reports stating that Nutrisphere-N has no effect on urease activity in urea-fertilized soils in the pH range 5.9 to 7.3 (Franzen et al., 2011) or 6.5 to 8.1 (Goos, 2013a). At the same time, these experiments provided the observation of urease enzyme inactivation at pH 5.0, an effect comparable to that observed for NBPT at the same pH (Fig. 4). The single monomers composing MIPs (maleic and itaconic acid) do not have any effect either at pH 7.5 (not shown) or at pH 5.0 (Fig. 3 and 4), suggesting that the presence of multiple carboxylate functional groups on the polymeric material is responsible for the observed effect. This indicates that a chelate effect is required for the urease inactivation process to occur, which facilitates extraction of the essential Ni(II) ions from the active site of the enzyme, with its consequent irreversible inactivation. This hypothesis is supported by measurements of the much larger amounts of Ni (as aqueous ions or bound to the small molecular weight MIPs) present in urease solutions incubated at pH 5.0 in the presence of MIPs as compared to the amount of Ni lost from urease at the same pH in the absence of MIPs. It is well-known that urease inactivation occurs at pH ≤ 5.0 through the generation of the apo-enzyme from the holo-enzyme because of the protonation of slightly basic amino acid residues bound to Ni(II) in the active site (Dixon et al., 1980). This process, in the presence of a parallel reaction of Ni(II) binding to MIPs, becomes more spontaneous, leading to inactivation rates comparable to those of NBPT, thus far the most widely used urease inhibitor in agricultural applications. The slightly better efficiency of BC as compared to T5 co-polymers correlates well with their different composition: BC is composed of only maleic and itaconic acids as monomeric units, while T5 contains up to 5% of additional components of the polymer made of sulfonate units, which have a much smaller propensity toward metal ion coordination and chelation.

The MIPs applied as a coating to urea granules prior to soil application are strongly acidic (pH 1 to 2), and the pH in the immediate surroundings of the granule, in the soil micropore, can easily reach values for which the Ni(II) loss from urease is spontaneous. Therefore, it can be envisioned that MIPs inactivate soil urease in a two-tiered process: while they provide the low pH necessary for the spontaneous release of Ni(II) ions from the active site of urease, leading to irreversible inactivation of its catalytic function. This effect, although not significant at pH 7.5, is very strong at pH 5.0, in conditions that can be easily achieved in the immediate surroundings of the MIPs-coated urea granules when applied in soil. The advantage of this process can also be regarded as affecting only a minor section of the fertilized soil, with small impact on the soil ecosphere, but the most important locus for its urease-inactivation action. Further developments are now expected in MIPs formulations that, in addition to the Ni-sequestration role at pH 5.0, could also contain known urease inhibitors able to further decrease the N loss due to ammonia volatilization at pH values closer to neutrality, with significant

**CONCLUSIONS**

The results obtained in the present study offer a novel perspective to the use of MIPs in effort to reduce ammonia volatilization following fertilization with urea. The mode of action that is supported by this study involves the role of MIPs as Ni-sequestering agents that facilitate the loss of the Ni(II) ions from the active site of urease, leading to irreversible inactivation of its catalytic function. This effect, although not significant at pH 7.5, is very strong at pH 5.0, in conditions that can be easily achieved in the immediate surroundings of the MIPs-coated urea granules when applied in soil. The advantage of this process can also be regarded as affecting only a minor section of the fertilized soil, with small impact on the soil ecosphere, but the most important locus for its urease-inactivation action. Further developments are now expected in MIPs formulations that, in addition to the Ni-sequestration role at pH 5.0, could also contain known urease inhibitors able to further decrease the N loss due to ammonia volatilization at pH values closer to neutrality, with significant
improvements of agricultural practices and reduced environmental impact. Even though the experiments were conducted in vitro and not using any soil, the chemistry of the enzymatic inhibition can be considered generally applicable, independently of soil properties such as organic matter content, water content, pH, composition or granulometry. In addition, this type of study allowed us to compare the results of similar in vitro experiments on the effect of MILPs on urease activity at neutral pH (Goos, 2013a). More detailed studies using soils with a range of properties, or more general agronomic tests, are necessary to investigate the effect of MILPs under field conditions.

REFERENCES


