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Graphical Abstract

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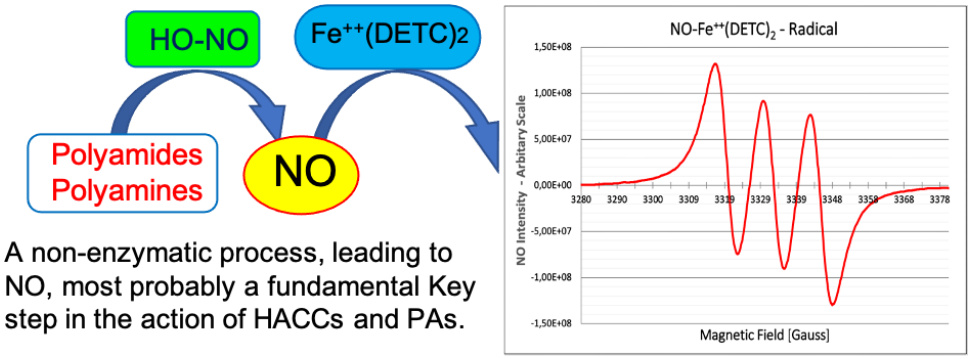

The crucial role of non-enzymatic NO-production in plants.

An EPR study.

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ABSTRACT

Polyamines and polyamides have a fundamental role in the biology of plants, and the presence of NO seems compulsory to account for their actions. In general, the NO production has claimed to occur through an enzymatic process, but not involving polyamines and polyamides. Nevertheless, a non-enzymatic mechanism, such as an electron transfer process among polyamines or polyamides and an acid nitrite solution, could account for rapid production of NO, even in anoxic conditions. EPR experiments, carried out with these substrates, proved the formation of NO. This evidence supports a non-enzymatic mechanism as an alternative source of NO, even in plants. So, since the NO production seems directly dependent on polyamines or polyamides presence, and these responsible for many activities in plants, it comes plausibly to consider crucial the involvement of NO in their actions. Furthermore, as for mammals, these results would confirm that, even in plants, NO production can occur through both enzymatic and non-enzymatic mechanisms.

Keywords: Polyamide, Polyamine, Nitric Oxide, EPR spectroscopy, Antioxidant, Antimicrobial

1. Introduction

The involvement of Nitric Oxide (NO), first identified in mammalian systems, was discovered in plants soon after (Delledonne et al., 1998; Kolbert et al., 2019), and quickly its specific role became evident (del Río et al., 2004; Qiao and Fan, 2008). Several distinct mechanisms of reaction, located in various cellular compartments and whose activation depends on different conditions, could be involved in NO production. At first, studies to discover NOS-type's mechanisms were prevalently conducted, but very soon even non-enzymatic mechanisms became of interest (Yamasaki and Cohen, 2006). NO seemed to be involved in a wide selection of processes (Neill et al., 2003; Mohn et al., 2019; Gupta et al. 2020), such as an antioxidant and an antiapoptotic modulator or a regulator of plant growth and development, as the response to plant stress and defence against pathogens, i.e., leading roles in plant defence (Besson-Bard et al., 2008). So, to cope with this abundant NO request, a non-enzymatic mechanism (Beligni et al., 2002) could be involved and have a pivotal role; therefore, as in mammalians (Jansson et al., 2001; Weitzberg and Lundberg, 1998), rapid availability of NO could also be required for plants' actions. In general, an enzymatic process is a multistep process and most likely not very fast; the only available, and rapid alternative source of NO, besides the possible accumulation from the environment (Popova, 2010), is an endogenous, non-enzymatic process. Furthermore, the NO synthase needs oxygen to work out but, under certain circumstances, the oxygen access to plant tissues is limited, and an alternative mechanism seems mandatory. For example, this can happen in roots where available oxygen is low (Stohr et al., 2001), and therefore a non-enzymatic process, independent of the oxygen, involved. This process could occur through the interaction of an acid nitrite (HO-NO) solution with mild reducing agents, such as specialized metabolites, and lead to the NO production via an Electron Transfer (E.T.) mechanism (Yamasaki, 2000). Nitrite is present in cells due to its accumulation when the photosynthetic electron transport is absent or inhibited, and the enzymatic production of NO (nitrate reductase) inactive (Yamasaki, 2000; Klepper, 1990). It follows that apoplastic nitrite might convert into NO via an E.T. process. To this aim, polyamides (HCAAs) and polyamines (PAs) could act as reducing agents, as supported by the rapid NO production and the increase in antioxidant capacity evidenced when adding exogenously PAs (Peng et al., 2016). So, determining the relationship between HCAAs/PAs and NO, to which a wide selection of regulatory functions is attributed, was a challenge.

2. Results and Discussion

2.1. Polyamides, Polyamines and Hydroxycinnamic Acids

Several varieties of flowering plants unveil the presence of different hydroxycinnamic acids conjugated with polyamines, for instance, feruloyl and caffeoyl putrescine, diferuloyl-putrescine, diferuloyl-spermidine, diferuloyl-spermine, p-coumaroylspermidine, feruoylputrescine, and caffeoylspermine, all present in ample amount in several higher plants (Takahashi and Kakehi, 2010;

Martin-Tanguy et al., 1978; Martin-Tanguy et al., 1979; Tiburcio et al., 2014; Kumar et al., 1997). Our research aimed to ascertain the mechanism of NO production and to understand the different abilities of HCAAs and PAs in inducing such a process, i.e., clarifying the role of the chemical structure of these substrates. In particular, for natural products such as HCAAs, PAs, and cinnamic acids (CAs), many are the studies addressed to clarify their different redox capacity, from different points of view. Some fundamental studies have tried to relate the antioxidant strength of natural products, for example, flavonoids, with their reducing capacity, which is strongly influenced by the type of phenolic groups present in the structure. (Jovanovic et al., 1994: Jovanovic and Simic, 2000; Clifford, 2004; Crozier, et al., 2009; Teixeira, et al., 2013). Therefore, since the phenolic/hydroxycinnamic acid radicals are present in the structure of all the polyamides examined, it is conceivable that these groups will influence the reducing activity of these species. So, since different is the reducing strength, different will be the efficiency in inducing the release of NO from the acid nitrite, and then the amount of NO trapped. In support of this, previous experiments conducted on hydroxycinnamic acids (Grossi and Casadei, 2020) such as ferulic acid (**7**), caffeic (**8**), and p-coumaric (**9**) (see Table 1), have shown the ability to induce the release of NO, through an E.T. process, from a solution of acid nitrite. A similar mechanism could explain NO production also in plants (Yamasaki and Cohen, 2006). Thus, by reacting a $NaNO_2$ acidic buffer solution (pH = 6.4) with HCAAs/PA/CAs, the released NO can be trapped through $Fe^{++}(DETC)_2$, and the paramagnetic NO adduct, NO-$Fe^{++}(DETC)_2$ thus formed, easily detected and identified by EPR spectroscopy (Grossi, 2014). This procedure had been previously used to detect NO in plants but, in those experiments, the spin-trap was directly introduced into the reaction pot (Caro and Puntarulo, 1999: Jasid, et al., 2006; Corpas, et al., 2004), and this changing the focal point of the process due to the reducing action of the trap (iron Fe^{++}), which will contribute to the release of NO. To avoid this, we conducted our EPR experiments with aqueous solutions of HCAAs and an acid nitrite buffer solution (pH = 6.4), keeping the spin trap solution separate, i.e., in a different vial as described in the experimental section (Qiao et al., 2008; Grossi and Casadei, 2020; Cevahir et al., 2007; Choi et al., 2007; Takahashi et al., 2003). **Table 1**.

2.2. Polyamides

By EPR, the amides deriving from putrescine and caffeic, ferulic, and p-coumaric acids, **1**, **2**, and **3** (see Table 1), were initially studied: all allowed the detection of the paramagnetic adduct, NO-$Fe^{++}(DETC)_2$, **Figure 1**.

Analogously, when experiments with spermidine amides deriving from cinnamic, p-coumaric, and ferulic acids (**4**, **5**, and **6**), were conducted, the formation of the paramagnetic adduct, NO-$Fe^{++}(DETC)_2$, was detectable, **Figure 2**.

These results confirmed the different ability of polyamides (HCAAs) to induce the release of NO, and this outcome, most probably, helps to understand the possible role of each polyamide in plants. Therefore, by comparing their EPR spectra (same scale, X and Y), since the intensity of the EPR

signals is proportional to the amount of NO detected/released, the different ability of each substrate to induce NO release becomes evident: the caffeoyl putrescine (**1**) resulted in the most efficient as shown in the inset pie chart, **Figure 3**.

2.3. The role of Amino and Hydroxycinnamic radicals

Such results underlined the strong dependence of the NO release process (efficiency) on the chemical structure of HCAAs, but not if one of the two radicals, amino or polyphenolic, present in the structure is more influential. To evaluate this, a comparison between the parents of HCAAs, amines, and hydroxycinnamic acids, could be helpful (Balasundram et al., 2006) since all these substrates can induce NO release through an E.T. mechanism. To this aim, the putrescine (**10**), as the reference amine, was studied under the same experimental conditions used for putrescine amides **1**, **2**, and **3**, and the respective EPR spectra reported in the same graph (same scale) and compared. Unexpectedly, the putrescine's signal was almost unnoticeable, and only after expanding the graph's Y-axis (out of scale) became evident (Fig. 4). This stressed the lower ability of amines (Besson-Bard et al., 2008; Balasundram et al., 2006) compared to amides (Cevahir et al., 2007; Tun et al., 2006) in inducing NO release. Inset, pie chart, **Figure 4**.

Finally, it was necessary to verify the role of hydroxycinnamic radicals. Results previously obtained in our laboratory (Grossi and Casadei, 2020) on hydroxycinnamic acids, such as caffeic (**7**), ferulic (**8**), and p-coumaric (**9**) could be used as a reference, allowing a comparison with putrescine-amides **1**, **2**, and **3**, under the same experimental conditions. The spectra we obtained, plotted on the same graph, showed amides to have a greater ability to influence the release of nitric oxide than pure hydroxycinnamic acids, **Figure 5**.

These experiments provided evidence that both amino and hydroxycinnamic radicals play a role in NO release but with different efficacy; therefore, it was useful to determine which of the two substrates was the most effective. To this purpose, a cross-check among the caffeoyl putrescine (**1**), the most effective substrate, caffeic acid (**7**), and putrescine (**10**) was carried out. EPR spectra showed the amide (**1**) as the most efficient NO inducer, much more than the caffeic acid (**7**), whereas the putrescine (**10**), also in this study/comparison, resulted almost negligible. Most probably, a synergic positive role of the two radicals could account for the drastic increase of the caffeoyl putrescine's (**1**) efficiency. **Figure 6**.

3. Conclusion

The results we report underline the possible role of the HCAAs, and parent molecules, in inducing NO release via a non-enzymatic process, which might be considered fundamental even for plants. For example, it could be predominant in those pathways in which a rapid supply of NO is requested, such as during an emergency event, and the nitrite widely present in the plant system could provide the possible pool. Studies conducted by EPR spectroscopy, testing polyamides, polyamines,

and CAs as likely NO inducer, let to detect different quantities of trapped NO, NO-$Fe^{++}(DETC)_2$, depending on the substrate investigated. In principle, EPR spectra can allow the evaluation of the amount of NO released, thus letting to classify these compounds based on their efficiency. To this aim, quantities of trapped radicals, determined via integration of the EPR spectra, and normalized to the least efficient amide (**5**), were calculated; the different efficiency in inducing NO release is evident, as well as the role of the chemical structure of amino and cinnamic radicals, see **Table 2**.

For example, the amide (**1**) is more efficient than parent amine (**10**) by over two orders of magnitude. These specialized metabolites, such as HCAAs or Polyamines, are considered to be involved in many processes, like defence against pathogens, antioxidants, antifungals, constituents of flowering plants. But, also NO in plants can interact with different intracellular and extracellular targets, acting as an antioxidant and/or having an antiapoptotic modulating action to prevent cell death (Cevahir et al., 2007), or protect from oxidative stress. Therefore, nitric oxide, whose presence/formation is strictly dependent on HCAAs and PAs, could be responsible for the actions of amides and amines in plants, since it can act as an antiradical, antioxidant, antifungal, antimicrobial, and much more. In definitive, the involvement of a non-enzymatic process to produce NO can be considered as a new point of view and provide new possibilities in understanding essential mechanisms of action of amides and amines in plants.

4. Experimental Section

All experiments were conducted at room temperature with commercial products, or products (HCAAs) kindly supplied by associated research laboratories, at the highest degree of purity available. The $Fe^{++}(DETC)_2$, iron (II) N,N-diethyldithiocarbamate, the NO-trap, was synthesized as previously reported (Grossi, 2009; 2014). As shown in Scheme, by reacting an $NaNO_2$ acidic buffer solution with HCAAs/PA/CAs, the released NO can be trapped through $Fe^{++}(DETC)_2$, and the paramagnetic NO-adduct, NO-$Fe^{++}(DETC)_2$, easily detected and identified by EPR spectroscopy. The experiments were carried out using a device obtained connecting two vials, by a tiny PTFE tube, both equipped with a porous septum; the first vial was filled with 3.0 ml of the acidic buffer solution (pH = 6.4) of $NaNO_2$, (5.0 mM), and reacted, in each experiment, with the same quantity, $(1.14 \pm 0.02) \times 10^{-5}$ mole, of HCAAs or CAs, or amine (final concentration, 2.28 ± 0.10 mM); the second vial was filled with 3.0 mL of a CH_2Cl_2 solution of NO-trap, $Fe^{++}(DETC)_2$, (4.0 mM). All samples were prepared by bubbling pure N_2-gas into the first vial, in which HCAAs or PA or CAs react with HNO_2, then conveyed into a second vial containing the radical trap; this process satisfies both the need to mix the reagents and the removal of NO, followed by its entrapment.

4.1. EPR experiments

All EPR spectra were recorded at room temperature, 5 accumulated scans, with an X-band Bruker EMX-EPR spectrometer (Bruker, Germany). The experiments were conducted on samples of the trap-

solution in which NO is collected for 20 minutes, i.e., letting flow-through the N_2-stream coming from the first vial (reaction pot); a small aliquot of this trap-solution is introduced in a capillary tube, the sample-tube, and investigated. EPR spectra were analysed using the Win-EPR and Simfonia programs (Brüker), and the output handled using Microsoft Excel to calculate the area, double integration. The operating conditions for the EPR spectrometer were as follows: centre field 333.0 mT, scan range 10 mT, microwave power 2.00 mW, microwave frequency 9.482 GHz, modulation frequency 100 kHz, receiver gain 4.48×10^4, and time constant 163.84 ms. Both, the hyperfine splitting constant (1.28 mT), which represents the distance between the peaks of the first two lines of the spectrum, and the g-factor (2.0039), a dimensionless parameter calculated by comparison with the DPPH (known g-factor), were experimentally measured and validated by comparison with results previously reported in the literature (Grossi, 2009; 2014); to convert Gauss to mT (milliTesla), we used the following relationship: 10 Gauss = 1 mT. Concerning the EPR spectra, it needs to stress that a standard spectrometer report on the X-axis the Magnetic Field, Gauss, and on the Y-axis the intensity of the signal (first derivative) in a Numerical Scale: these are the parameters reported into the spectrum (output). In this study, only one radical species was investigated, always in the same experimental conditions, i.e., at room temperatures and with the same instrumental parameters: the radical species is likely in free tumbling (Carrington and McLachlan, 1969). Thus, the line shape will remain almost unchanged in all the experiments, allowing the radical quantitation by comparing the spectra area.

Author Contribution

The manuscript was written with the contributions of all authors, who read and approved the final version.

Competing Interest

The authors declare no competing financial interest.

Acknowledgements

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Figures

Figure 1.

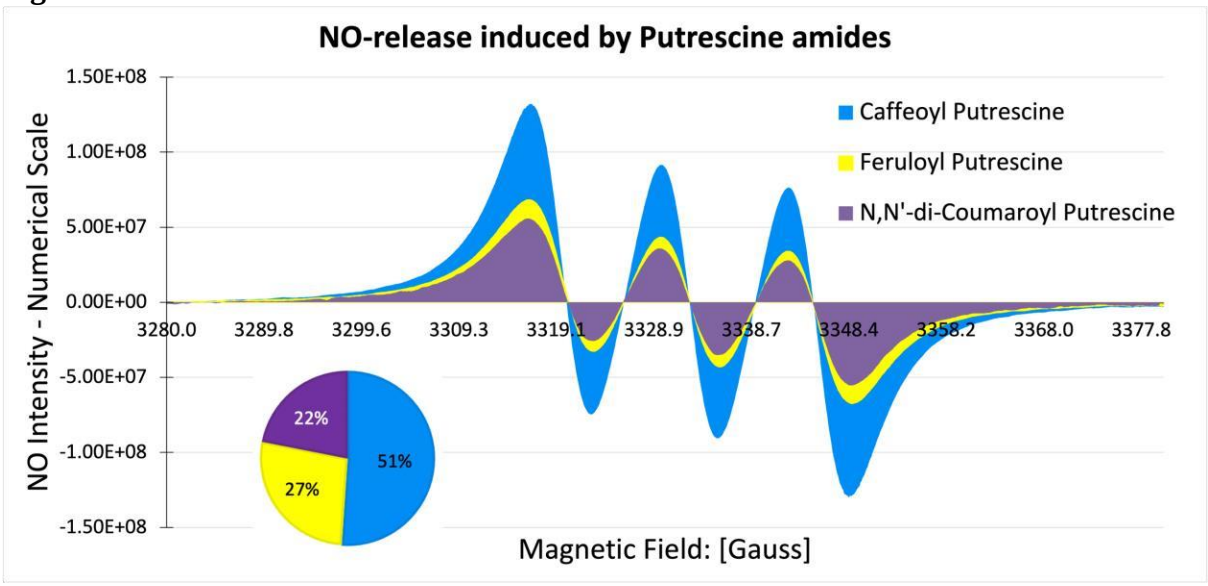


Figure 2.

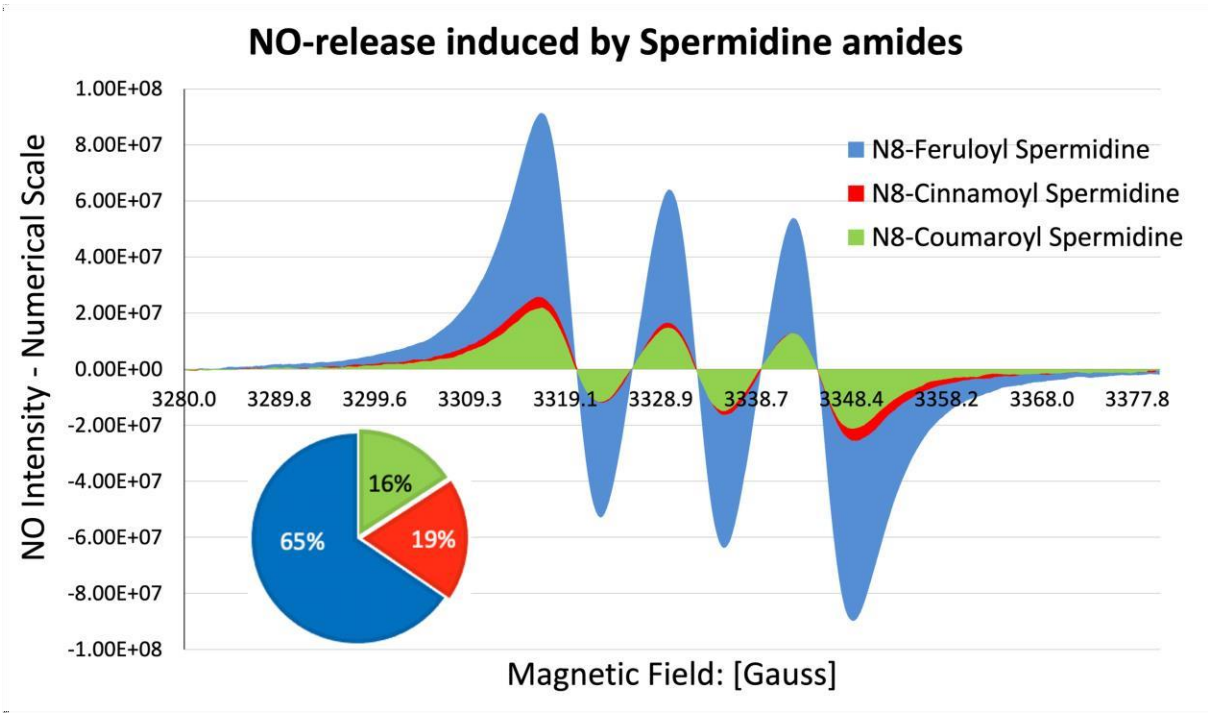


Figure 3.

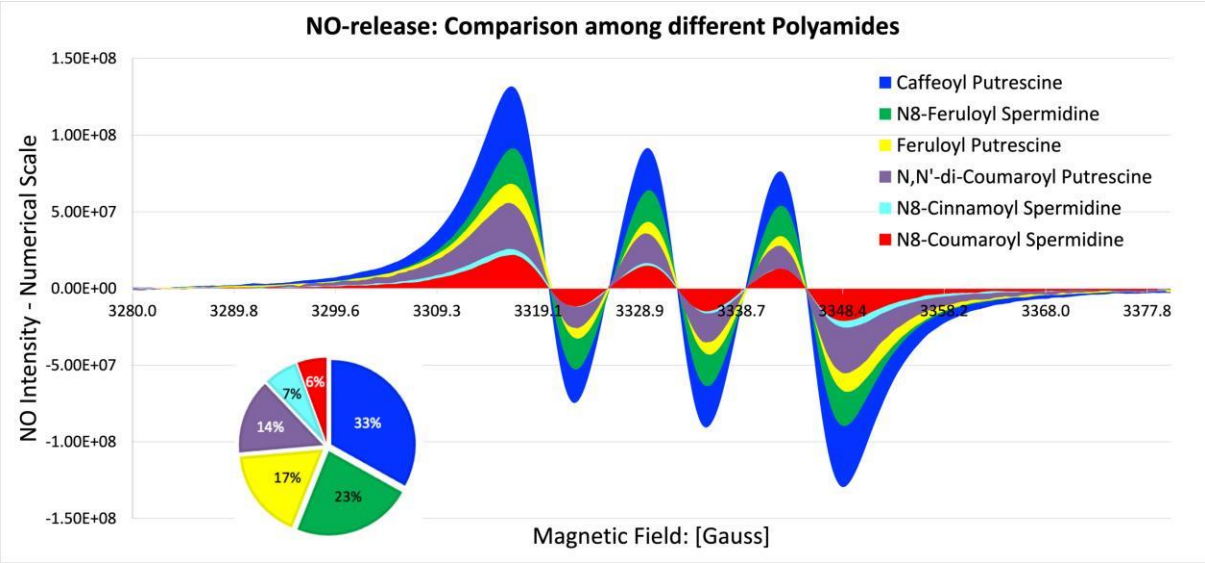


Figure 4.

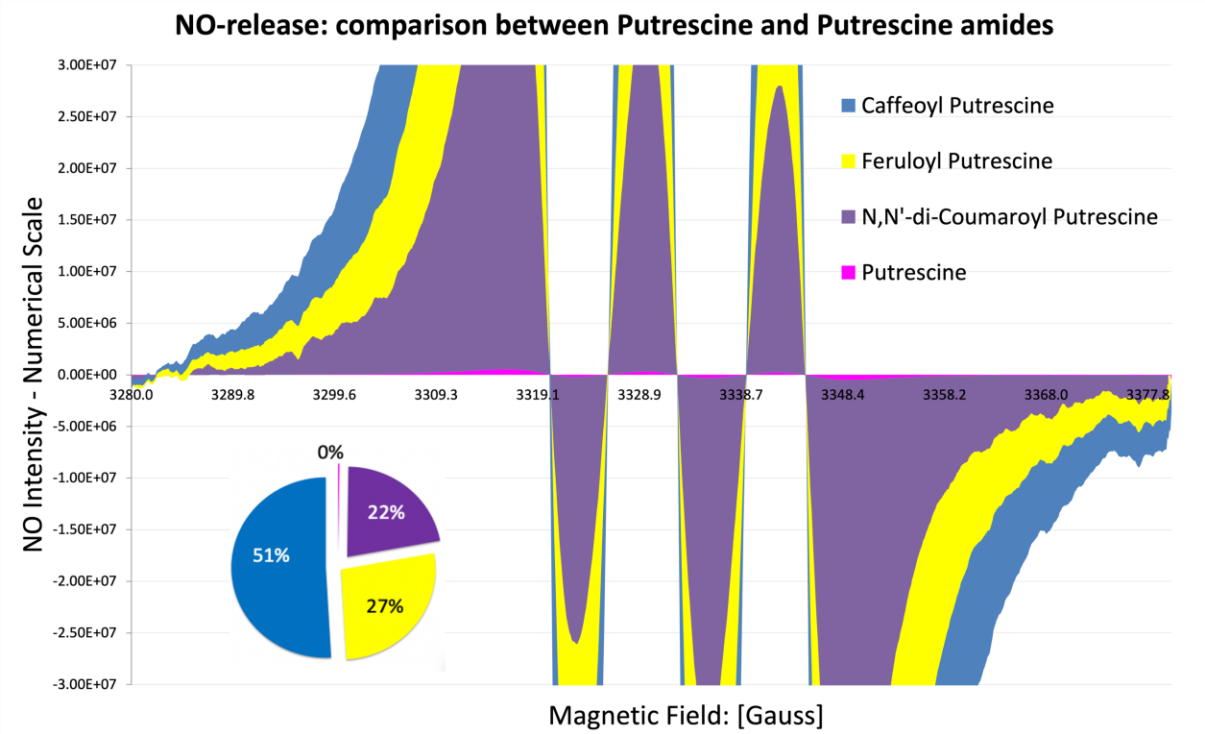


Figure 5.

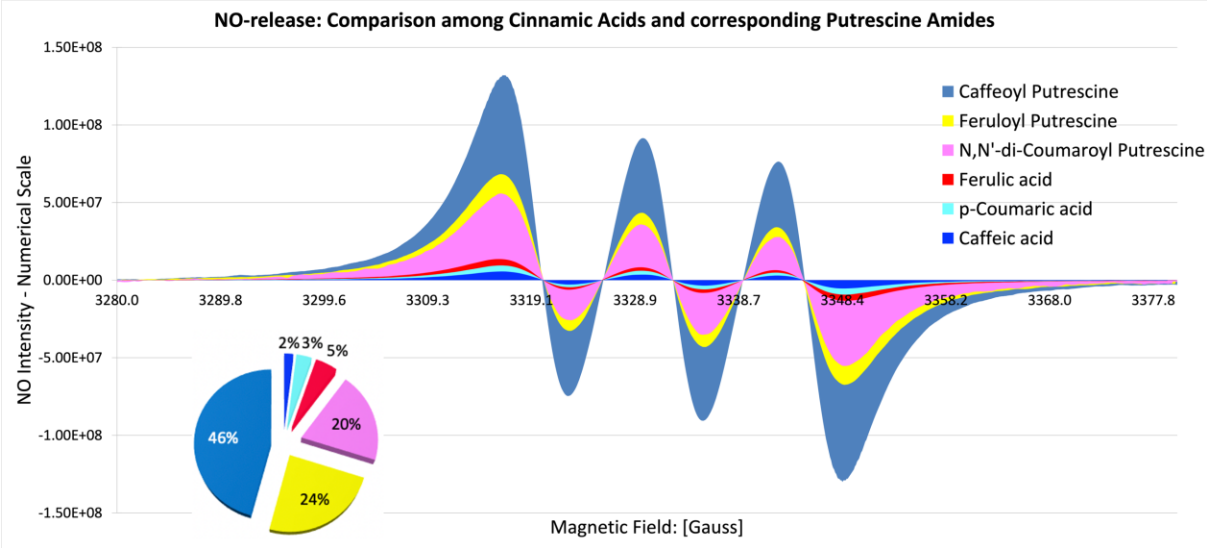
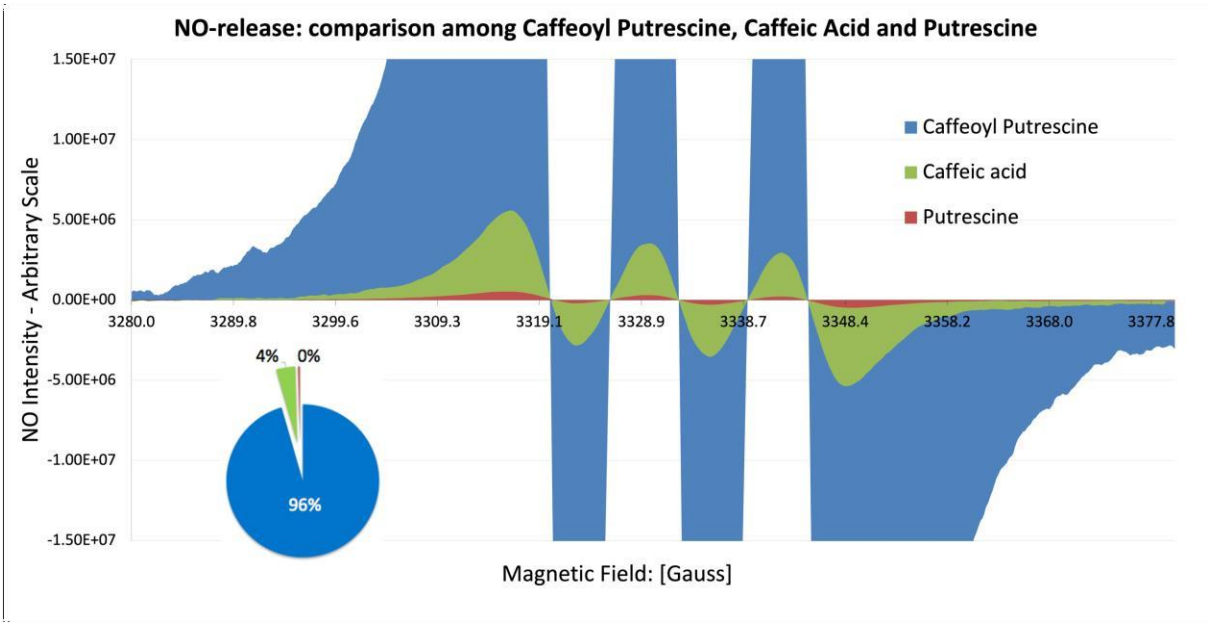


Fig. 6



Legends

Figure 1. NO-release induced by Putrescine Amides, **1**, **2** and **3**. EPR spectra of NO-$Fe^{++}(DETC)_2$, a_N =1.28 mT and g = 2.039. Inset: relative percentages of NO released. For all substrates the final concentration was 2.28 ± 0.10 mM.

Figure 2. NO-release induced by Spermidine Amides, **4**, **5** and **6**. EPR spectra of NO-$Fe^{++}(DETC)_2$, a_N =1.28 mT and g = 2.039. Inset: relative percentages of NO released. For all substrates the final concentration was 2.28 ± 0.10 mM.

Figure 3. NO-release: comparison among different Polyamides. EPR spectra of NO-$Fe^{++}(DETC)_2$, a_N =1.28 mT and g = 2.039. Inset: relative percentages of NO released. For all substrates the final concentration was 2.28 ± 0.10 mM.

Fig Figure 4. NO-release: comparison between Putrescine and Putrescine amides. EPR spectra of NO-$Fe^{++}(DETC)_2$, a_N =1.28 mT and g = 2.039. Inset: relative percentages of NO released. For all substrates the final concentration was 2.28 ± 0.10 mM.

Figure 5. NO-release: comparison among Cinnamic Acids and corresponding Putrescine amides. EPR spectra of NO-$Fe^{++}(DETC)_2$, a_N=1.28 mT and g = 2.039. Inset: relative percentages of NO released. For all substrates the final concentration was 2.28 ± 0.10 mM.

Figure 6. NO-release: comparison among Caffeoyl Putrescine, Caffeic Acid and Putrescine. EPR spectra of NO-$Fe^{++}(DETC)_2$, a_N=1.28 mT and g = 2.039. Inset: relative percentages of NO released. For all substrates the final concentration was 2.28 ± 0.10 mM.

TABLES

Table 1. Hydroxycinnamic Acids Amides, Hydroxycinnamic Acids, and Amines investigated.

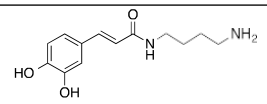
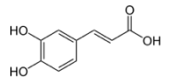
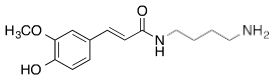
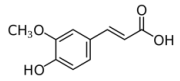
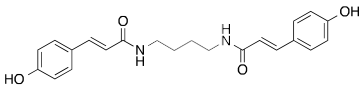
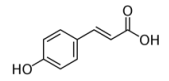
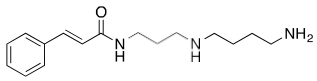
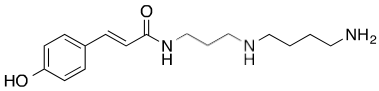
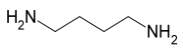
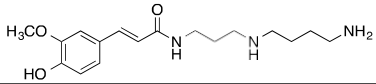
Hydroxycinnamic Acids Amides		**Hydroxycinnamic Acids**	
(**1**) Caffeoyl putrescine		(**7**) Caffeic Acid	
(2) Feruloyl putrescine		(**8**) Ferulic Acid	
(3) N,N'- di-Coumaroyl putrescine		(**9**) p-Coumaric acid	
(4) N8-Cinnamoyl spermidine		**Amine**	
(**5**) N8-Coumaroyl spermidine		(**10**) Putrescine	
(**6**) N8-Feruloyl spermidine			

Table 2. Relative efficiency in inducing NO release. Comparison among HCCs, CAs, and PA.

Compared Substrates	Area of EPR Spectra* Numerical Scale	Relative amounts of NO released, normalized to (**5**). Numerical Scale
(**1**) Caffeoyl Putrescine	3.551×10^9	**5.95**
(**6**) N8-Feruloyl Spermidine	2.465×10^9	**4.13**
(**2**) Feruloyl Putrescine	1.883×10^9	**3.15**
(3) N,N'- di-Coumaroyl Putrescine	1.527×10^9	**2.55**
(**4**) N8-Cinnamoyl Spermidine	0.705×10^9	**1.18**
(**5**) N8-Coumaroyl Spermidine	0.597×10^9	**1.00**
(**8**) Ferulic Acid	0.375×10^9	**0.63**
(**9**) p-Coumaric acid	0.259×10^9	**0.43**
(**7**) Caffeic Acid	0.152×10^9	**0.25**
(**10**) Putrescine	0.015×10^9	**0.025**

*EPR spectra area are related to the detected radical concentration.

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Figure

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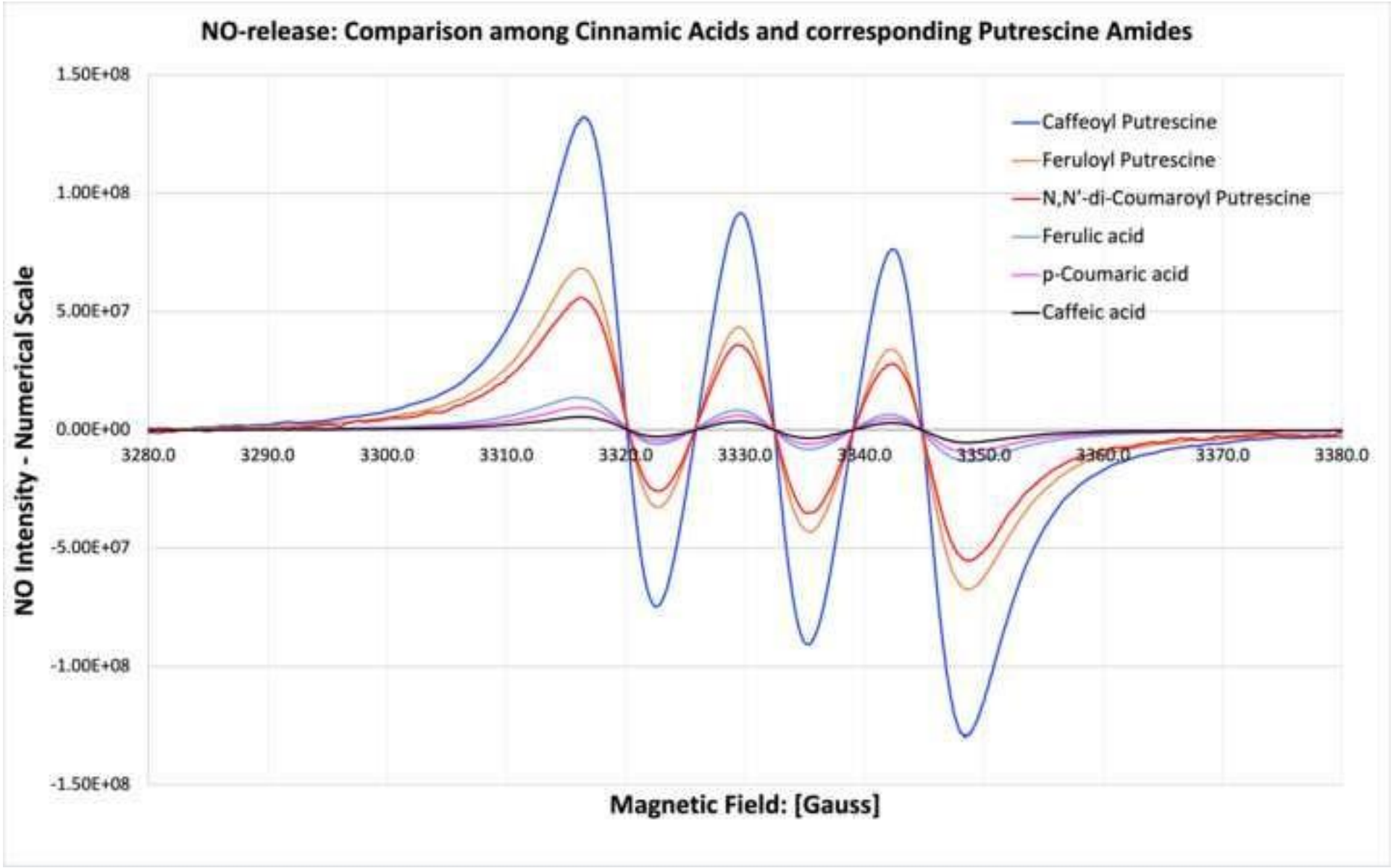
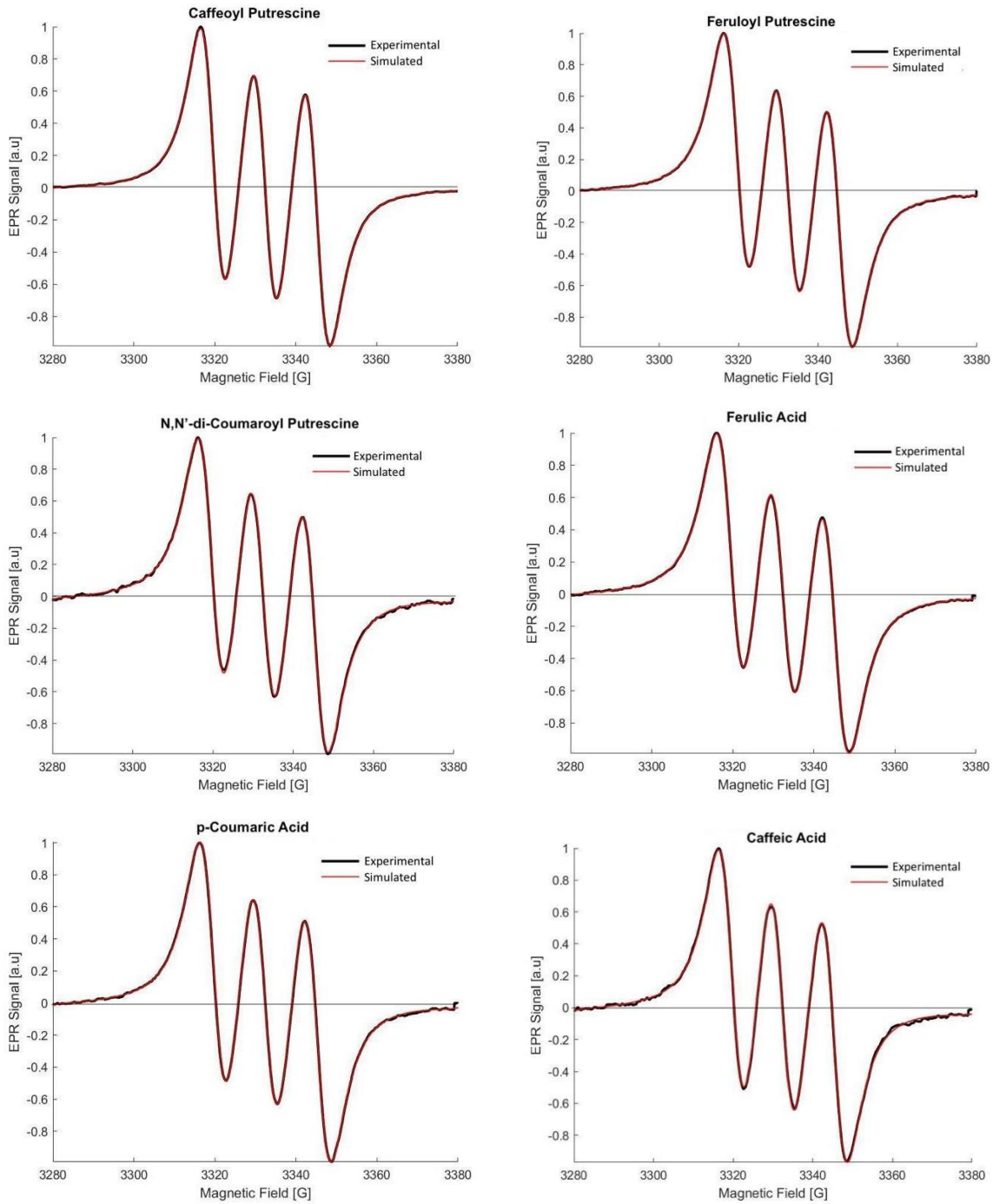


Table - Supporting Information

Table: Evidence of the excellent overlap of the line shape of spectra simulated (red line) with the experimental ones (black line).



No conflict of interest to declare.