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Original Article

N-methyl-2-phenylindole Colorimetric Method as Biomarker for Lipid Peroxidation in *Pisum sativum*

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Abstract

A novel and selective method to quantify lipid peroxidation in *Pisum sativum* by measuring one of its secondary products, malondialdehyde (MDA), by its reaction with *N*-methyl-2-phenylindole was validated in this study. It has been reported that indirect effect of heavy metals in plant macromolecules via reactive oxygen species (ROS) production is more toxic and fast than its direct effect. The OH• is produced by metal-catalized Fenton reaction, as a consequence, metal presence in soils may potentiate lipid peroxidation. Enzymes such as cyclooxygenase and lipoxygenase may initiate lipid peroxidation too, therefore, either ROS scavenging or lipoxygenases (LOX) or cyclooxygenases (COX) inhibition is convenient routes to diminish lipid peroxidation occurrence. Two-way analysis of variance (ANOVA) was performed to determine if interaction between time and treatment result in a significant difference between MDA levels in 12 plants. Bonferroni test revealed that treatment was not consistent with time. The day that resulted in significant difference of MDA was the eighth day. MDA to perform the reaction was extracted from groups of twelve 8-day plants by sonication in hydrochlorhydric acid. A calibration curve using 1,1,3,3, tetramethoxypropane as MDA standard, HCl 1 N in methanol, acetonitrile-methanol (3:1) as solvent was performed. Its linear range was 0.0078 µM to 1 µM with a 99.8% correlation. Sensibility of the method was enhanced by variation of time and temperature. Repeatability reported as variation coefficient yielded 7.8% for the standard curve and 19.6% for the samples, being these latter representative of combined biological and chemical variability. A natural flavonoid rich fraction extracted from *Siparuna gigantotepala* and a synthetic pyrimidine-type compound were evaluated as potential lipid peroxidation inhibitors. Flavonoid rich fraction inhibits the reaction by scavenging radicals while pyrimidine-type compound inhibits LOX and COX enzyme-catalized lipid peroxidation. Both potential inhibitors showed lipid peroxidation inhibition as evidenced by returning MDA concentration in stressed plants to that in control plants.

Keywords: Lipid peroxidation, Pisum sativum, N-methyl-2-phenylindole method, Malondialdehyde, Flavonoids, Oxidative stress

Introduction

Land and soils constitute the foundation for sustainable agricultural development, essential ecosystem functions and food security. The recent increase of heavy metals in agricultural soils is a threat for this latter since heavy metals are involved in early stage toxicity induced by heavy metals in crops and act as initiators of reactions like lipid peroxidation.[1,2] Metals induce this reaction by increasing reactive oxygen species (ROS) generation.[3] ROS include both, free radicals such as $O_2^{\cdot-}$, superoxide radical, hydroxyl radical, alkoxy radical and molecular forms nonradical like H_2O_2 and $^1O2$ singlet oxygen.[4] When a hydroxyl ion attacks a polyunsaturated fatty acid (PUFA), cell membrane is also altered causing a dramatic effect on cell integrity.[5] Thus, research of synthetic and natural agents to mitigate effects of lipid peroxidation has grown. Lipid peroxidation may occur by ROS or enzymes like cyclooxygenases (COX) and lipoxygenases (LOX),[6] accordingly, potential inhibitors may either capture ROS or inhibit COX, LOX enzymes.

On the other hand, lipid peroxidation has been measured by several methods quantifying either its primary products, hydroperoxides, or its secondary products, alkenals, conjugated dienes, and malondialdehyde (MDA). The thiobarbituric acid (TBA)[7] method, the most commonly used, has an associated limitation to the fact that other aldehydes and other substances like protein, sucrose, and urea may react with TBA[7,8] generating colored species and

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consequently contributing to over estimation in lipid peroxidation measure.[9] To avoid these issue, the validation of a novel and selective method to quantify MDA with *N*-methyl-2-phenylindole in presence of HCl[10] (Figure 1) was achieved to assess lipid peroxidation in *Pisum sa-tivum*[3,11] exposed to copper and to determine inhibitory activity of 2 potential inhibitors: a flavonoid rich fraction of *Siparuna gigantotepala* and a synthetic pyridopyrimi-dine type compound, acid 3-methyl-2-methyltio-5-(4-methoxiphenyl)-4-oxo-3,4,5,8-tetrapirido[2,3-d]pyrimi-din-7-carboxilic.

Materials and Methods

Plant Material and Growth Conditions

Seeds of pea (*Pisum sativum*) were germinated as follows: First they were immersed for 3 hours in 0.2% w/v sucrose in water, washed 3 times with water and were half submerged in 15 mL water for 3-4 days in covered Styrofoam dishes with filter paper and then water germinated seed were propagated individually in growth pots filled with cottons. Seeds were grown under indoor lighting conditions, temperature (25°C) and watered (3-4 mL of water for 8 days). Batches of 12 seeds were subjected to one of the different treatments on the first day of sown: Firstly, to assess the best day for evaluation of lipid peroxidation, batches were processed at day 8 and day 13 using 2.0 mM copper sulfate to induce lipid peroxidation. Secondly, batches were treated with either 1.0 mM or 2.0 mM copper sulfate to induce lipid peroxidation and to determine the concentration of the transition metal that result in MDA content at a significant different level to control plants (grown only with water). Thirdly, batches of plants, where lipid peroxidation was induced by 2.0 mM copper sulfate, were harvested on day 8. They were treated in the first day with either 4 mL of 300 µg/mL rich flavonoid fraction from *Siparuna gigantotepala* or 4 mL of 400 µM a pyridopyrimidine type compound.

Calibration Curve Preparation

To avoid the over estimation of lipid peroxidation measure[9] in the TBA test due to the fact that other aldehydes and other substances like protein, sucrose, and urea may react with TBA[7,8] a more selective method to quantify MDA with *N*-methyl-2-phenylindol in presence of HCl[10] (Figure 1), producing a carbocyanide readable at 586 nm was standardized an validated.

The malonaldehyde bis(dimethyl acetal), 1,1,3,3-Tetramethoxypropane (TMOP) was used as standard. A 100 µM TMOP stock solution was prepared by dissolving 164 µL of the 99% purity reagent in a 10 mL volumetric flask, using acetonitrile as a solvent. With this solution, a 10 µM TMOP working solution was prepared by adding to 1 mL of it, 1.5 mL of 1M HCl and using acetonitrile-methanol 3:1 as a solvent in a 10 mL flask. A 2000 µM stock solution of *N*-methyl-2-phenylindole reagent was prepared, 414 mg were dissolved in 15 mL of HCl (37%), then 20 mL of deionized water were added and acetonitrile-methanol 3:1 was used as a solvent in a 100 mL flask. With this solution, a 200 µM chromogenic solution was prepared by adding to 1 mL of it, 1.5 mL of 1M HCl in methanol and using acetonitrile-methanol (3:1) as solvent.

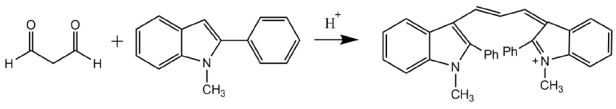

Figure 1. *N*-methyl-2-phenylindole Reaction With Malondialdehyde (MDA) and Carbocyanide Reaction Product.

Calibration curve was prepared in an eppendorf 96-well plate (1.5 mL) by 2-fold serial dilutions of 400 µL of 10 µM TMOP in acetonitrile obtaining a curve of TMOP concentrations between 0.0078–1.0 µM. Then, 40 µL of isopropanol, 80 µL of 1M HCl in methanol, 80 µL of 200 µM *N*-methyl-2-phenilindole reagent were added to each well, and 1 mL volume was reached in each well with 600 µL of 1M HCl in methanol. The eppendorf curve was incubated at 60°C for 3 hours and then 250 µL of each well were transferred to a brand microplate to read the absorbance in a microplate reader (Metertech AccuRead 960+) using a 600 nm filter.

Method Validation

The process of verifying if a method of *N*-methyl-2-phenylindole was suitable for the purpose of quantifying MDA in *Pisum sativum* consisted in evaluation of some parameters for method validation such as linear range, linearity, precision by repeatability and reproducibility, limit of detection (LOD) and limit of quantitation (LOQ), and sensibility.

Statistical analyses for method validation were performed using the software Graphpad Prism. In one-way analysis of variance (ANOVA) and two-way ANOVA analysis, results were assumed statistically different if $P < .05$. In the following text a single asterisk denotes significant, with *P* value between the .01 and .05, double asterisk denotes very significant, with *P* value between .001 and .01 and triple asterisk denotes extremely significant, with *P* value smaller than .001.

Lipid Peroxidation Evaluation

The MDA content was determined by the *N*-methyl-2-phenylindole reaction under HCl acidic conditions based on a research on mechanistic aspects of the colorimetric assay.[10] A 300 mg crushed sample was macerated and ultrasound homogenated for 15 minutes in 1 mL HCl 1N in 1.5 mL containers. The supernatant was centrifuged at 12000 rpm for 10 minutes. Forty microliters of isopropanol, 80 µL of HCl 1N, and 80 µL of the chromogenic reagent were added to 800 uL aliquot of the supernatant of each plantlet in an eppendorf 96-well plate (1.5 mL of volume). Incubation for 3 hours and a portion was used for measurement at 600 nm was read and the MDA concentrations in micromoles per gram of fresh weight were cal-

culated. TMOP and *N*-methyl-2-phenilindole were supplied from Sigma Aldrich (USA), methanol, acetonitrile, isopropanol from Merck, all products of analytic grade.

Results and Discussion

Method Validation

Three calibration curves (6 replicates) in 3 different days were prepared in the determined linear range. Curve linear fit was made by a nonlineal regression with automatic outlier elimination. A Student's *t* test was performed in the linear range to test correlation between TMOP concentration and absorbance lectures as shown in tables 1 and 2.

Linearity was achieved as shown by determination coefficients (r^2) greater than 0.996 for all calibration curves and correlation coefficient between absorbance and TMOP concentration by a Student's *t* test. The test showed a significant correlation between concentration and absorbance since r was positive and *P* was small, with 95% confidence level for the interval 0.0078 to 1 µM TMOP.

To determine if the matrix of *Pisum sativum* had an effect on the reaction with the chromogenic and therefore and effect in the absorbance lecture, a calibration curve using addition standard method was performed as shown in Figure 2.

The curve was prepared as well by standard addition method using 250 µL of plant extract as fixed sample volume. Comparison of slopes by an F test ($P = .7695$) indicated that there are not significant differences between slopes, as a consequence, there are no matrix effect interferences. So, regular calibration quantitation gives correct values for MDA in *Pisum sativum*.

N-methyl-2-phenylindole reaction test was standardized and validated to quantify MDA in *Pisum sativum* as lipid peroxidation biomarker. Evaluation of linearity in regular calibration curve was achieved with r^2 of 0.9986 and 3 calibration curves prepared independently. Since regular calibration curve and standard addition method curve present no significant difference by an F test, regular calibration may be used for this method, no matrix effects were found, assuring correct values in quantitation of MDA in

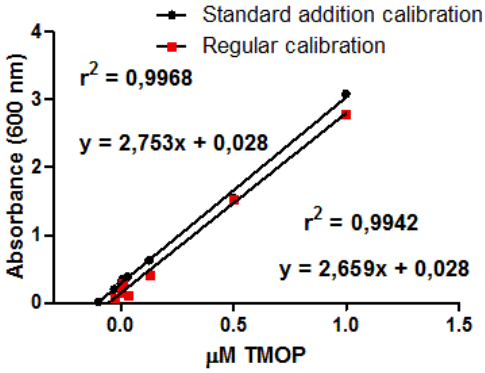

Figure 2. Calibration graphs by regular calibration and standard addition method for microplate assays of *N*-methyl-2-phenylindole method to quantify malondyaldehyde (MDA).

Pisum Sativum for regular calibration.

Precision was determined by repeatability and reproducibility. Standard deviation concentration for 6 replicates at each concentration, relative standard deviation and repeatability limit are shown in Table 3. Relative standard deviation of 12 plants for MDA quantitation according to each treatment is shown in Table 4.

The total variation coefficient is 7.8%. It was considered that method repeatability was acceptable being the variation coefficient less than 10%. For MDA quantitation, total variation coefficient was 19.6% which is greater than that of calibration curve, but taking into account of biological and chemical variability is acceptable.

Repeatability limit, the value less than or equal to which the absolute difference between 2 test results obtained under repeatability conditions may be expected to be with a probability of 95%. It was calculated by the following formula where *t* is the Student's 2-tailed value for 95% confidence where the value is 1.96), and σ_r is the standard deviation measured under repeatability conditions.

$$r = t \times \sqrt{2} \times \sigma_r$$

As for repeatability, it does not include the minimum concentration, which has a difference between duplicate analyses larger that the repeatability limit. Thus, repeatability was achieved for standards 2 to 8, the method is consid-

Table 1. Results for *T* Test for Correlation of TMOP Concentration and Absorbance Lectures

Compound	r	P (1-tail)
TMOP	0.9972	<.0001

Abbreviation: TMOP, 1,1,3,3-Tetramethoxypropane.

Table 2. Determination Coefficients and Curve Equation of 3 Calibration Curves Prepared Independently

Calibration Curve	r^2	Curve Equation
1	0.9982	y = 2.712x + 0.0154
2	0.9985	y = 2.866x + 0.0239
3	0.9961	y = 3.045x + 0.0357
Average	0.9986	y = 2.895x + 0.0210

Table 3. Results for Standard Deviation, RSD and Repeatability Limit for 8 Standards (6 Replicates of Each)

	Standard Deviation	RSD (%)	r[a]
Standard 8	0.091	3.1	0.252
Standard 7	0.066	4.5	0.183
Standard 6	0.035	4.5	0.096
Standard 5	0.035	8.2	0.096
Standard 4	0.013	6.4	0.037
Standard 3	0.013	12.3	0.036
Standard 2	0.005	9.5	0.015
Standard 1	0.005	14.3	0.0129

Abbreviation: RSD, Relative Standard Deviation.
[a] r=repeatability limit.

Table 4. Relative Standard Deviation Values of MDA at 8 or 13 Days

Treatment	Day 8	Day 13*
0.0 mM Cu^{2+}	16.6	13.2
1.0 mM Cu^{2+}	19.4	N.A.
2.0 mM Cu^{2+}	29.3	24.5
2.0 mM Cu^{2+} + 300 ug/mL Rich-flavonoid fraction	13.6	N.A.
2.0 mM Cu^{2+} + 400 mM pyrimidine-type	20.5	N.A.

Abbreviation: N.A., Not applicable.

ered repetitive.
Comparison of slope and intercept of calibration curves 1 and 2 prepared on different days showed that the method reproducibility over time was acceptable since differences were not significant on these parameter, F-test *P* value of .59.
LOD and LOQ were determined according to Eurachem guide.[12]
Same spectrophotometric reaction was carried out with-out TMOP (blank) in 10 replicates and measured the ab-sorbance at 600 nm, then the mean of these absorbance is found "b".

Absorbance (p) = mean of blank + 3SD (LOD).

Absorbance (q) = mean of blank + 10SD (LOQ).

LOD was calculated as the analyte concentration of absor-bance p, 4,081E-3 µM TMOP. LOQ was calculated as the analyte concentration of absor-bance q 0,00136 µM TMOP.

Sensitivity is the measure of the change in the instruments' response that corresponds to a change in the analyte concentration. An increase in sensitivity was achieved by variation of temperature and time conditions with respect to those reported by Erdelmeier et al.[10] In Figure 3, calibration curves (6 replicates per standard) with initial and final temperature and incubation time conditions are shown. As observed in slope of calibration curve with final con-ditions, an improvement in sensitivity in a factor greater than 10 times more sensible was achieved in the develop-ment of the method.

Lipid Peroxidation Evaluation

In quantitation, by a one-way ANOVA and a Tukey test, results indicate that plants treated with 2.0 mM copper sulfate solution present a difference at a significant level ($P < .001$) with respect to control plants in MDA content per gram of fresh weight of plant. In contrast, samples treated with 1.0 mM copper sulfate solution did not present a difference at a significant level with respect to control plants (see Figure 4). Thus, treatment with copper sulfate 2.0 mM was chosen to establish an oxidative stress treatment that induces lipid peroxidation in a marked fashion. Results of two-way ANOVA and Bonferroni post test show that the effect of treatment is not the same if plants are harvested at day 8 or at day 13. Since difference at a significant level of MDA quantified is present at day 8 (see Figure 5), this day was chosen for harvesting plants to analyze potential of inhibitors of lipid peroxidation (Figure 3 and 4).
To choose potential inhibitors of oxidative stress due to lipid peroxidation, inhibition suggested mechanisms for this reaction were taken into account. Flavonoids protect lipids against oxidative damage by several mechanisms. Flavonoides are thermodynamically able to reduce free radicals with high oxidant, the compounds will inhibit generation of free radicals by chelation ions (iron, copper, etc).[3] Results for treatment with the flavonoid rich fraction are shown in Figure 6.
Results for one-way ANOVA and Tukey show that plants subjected to treatment with copper sulfate 2.0 mM plus treatment with 300 µg/mL flavonoid rich fraction were not different at a significant level from control plants. Thus, the flavonoid rich fraction possibly affected inhibition of lipid peroxidation resulting similar values to the controls. Inhibition of lipid peroxidation may undergo by inhibition of LOX or COX enzymes that catalyze it. Inhibition of the former has shown to reduce the enzyme from its hydroxide bound ferric (active) form to its water-bound ferrous (inactive) form. Nam et al[13] have suggested a mechanism by which pyrimidine and pyridine type compounds inhibit LOX, by complexation of the iron via a ring nitrogen atom and the carbonyl oxygen of the acetamido group. In this way, inhibition of lipid peroxidation by pyrimi-

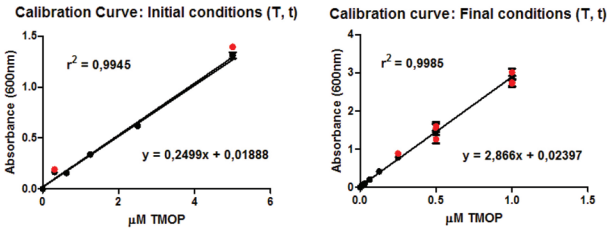

Figure 3. Calibration Curves Conditions. Left: with initial temperature (40°C) and incubation time (1 hour). Right: Calibration curve with final temperature (60°C) and incubation time (3 hours).

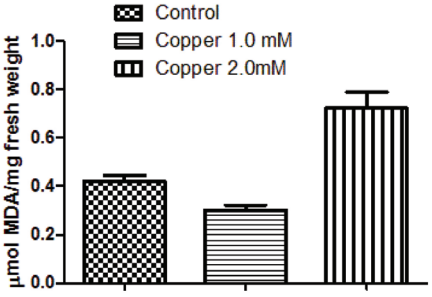

Figure 4. Comparative malondialdehyde (MDA) content (average and standard deviation) according to treatment: copper absence, 1.0 mM copper and 2.0 mM copper.

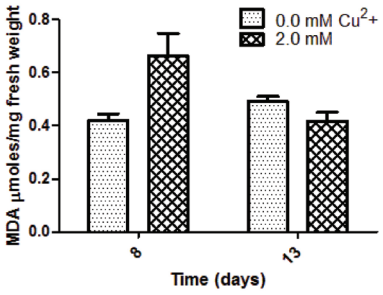

Figure 5. Results for the experimental design of 2 factors: Harvest time and treatment.

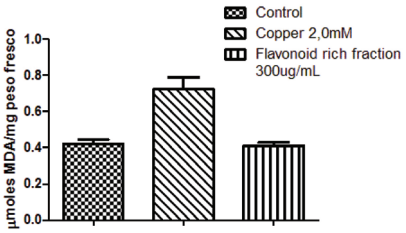

Figure 6. Comparative of malondialdehyde (MDA) content (average and standard deviation) according to treatment: Control, 2.0 mM copper sulfate, and 2.0 mM copper sulfate exposed to 300 µg/L rich flavonoid fraction.

dine type compound acid 3-methyl-2-methyltio-5-(4-methoxiphenyl)-4-oxo-3,4,5,8-tetrapirido[2,3-d]pyrimidin-7-carboxilic may occur via LOX enzyme inhibition by an analog mechanism. Results for treatment with the pyridopyrimidine type compound are shown in Figure 7. Results for one-way ANOVA and Tukey analysis show that plants subjected to treatment with copper sulfate 2.0 mM plus treatment with pyridopyrimidine type compound were not different at a significant level from control plants. Inhibition of lipid peroxidation by the pyridopyrimidine compounds were affected by copper sulfate showing similar MDA values as for controls.

Conclusions

Sensitivity in quantification by regular calibration curve spectrophotometric method was improved by a variation of temperature and incubation time conditions regarding to those reported in literature for the reaction of *N*-methyl-2-phenyindole with MDA in HCl acidic conditions. Reproducibility and repeatability were assessed with acceptable variation coefficients taking into account calibration curve only and quantitation, in which a larger value explains both the biological and chemical variability.

A rich flavonoid fraction from *Siparuna gigantotepala* and a pyrimidine type compound showed lipid peroxidation inhibitory activity in *Pisum Sativum* exposed to copper as evidenced by reverting the production of MDA to that of control plants. These results suggest the *N*-methyl-2-phenylindole reaction test for determining MDA as a valuable spectrophotometric technique to search for lipid peroxidation potential inhibitors from natural and synthetic sources.

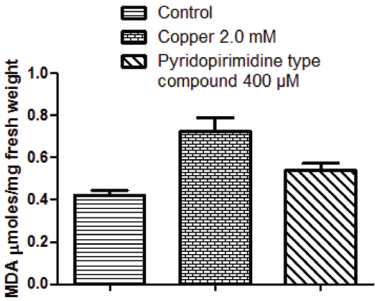

Figure 7. Comparative of malondialdehyde (MDA) content (average and standard deviation) according to treatment: Control, 2.0 mM copper sulfate, and 2.0 mM copper sulfate exposed to 400 µM pyridopyrimidine type compound.

Competing interests

The authors declare no competing interests.

Acknowledgments

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