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Analysis of Interfering Substances in the Measurement of Malondialdehyde Content in Plant Leaves

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ABSTRACT

The Thiobarbituric Acid (TBA) reactive substances assay is an easy and quick assay for the assessment in plants of lipid peroxidation, in which Malondialdehyde (MDA) is derivatized. To analyze the applicability of this method, the MDA concentrations in the leaves of different plants were measured by the TBA method. To further separate the interfering substances, fractional extraction and Gas Chromatography-Mass Spectrometry (GC-MS) were used in this research. The results suggest that lipid peroxidation is accurately measured by the TBA method with trichloroacetic acid as the extraction solution in most plants. However, the method was not suitable for measuring the MDA concentration in golden privet (*Ligustrum* × *vicaryi*) leaves. Negative MDA concentrations were obtained in golden privet leaves by this method. The interfering substance in golden privet leaves, which could react with TBA forming a dark green production (the characteristic absorption peak at 615 nm). This substance was highly lipid soluble and was found at the highest level in golden privet leaves. Compared with ether phase extracted from stems, four different compounds in golden privet leaves were found, which were similar to diethyl hydroxybutanedioate (similarity: 94%), 3,4-dimethoxybenzoic acid (72%) and quinuclidine-2-carboxylic acid, 2,3-dehydro-3-amino-ethyl ester (63%) and yramine, N-formyl- 2-(4-Hydroxyphenyl) ethylformamide (82%), respectively.

Keywords: Gas Chromatography-Mass Spectrometry, Interfering Substance, Lipid Peroxidation, Malondialdehyde, Thiobarbituric acid

1. INTRODUCTION

In plants, the toxic effects of abiotic and biotic stresses include a decrease in photosynthetic activity (Lim *et al*., 2007), a reduction in water and nutrient uptake, growth inhibition (Khalvati *et al*., 2010; Gutha and Reddy, 2008), damage to proteins (Mot *et al*., 2007) and changes in lipid composition (Novitskaya *et al*., 2004) and lipid peroxidation (Shah *et al*., 2001). Lipid peroxidation has been suggested to be responsible for the developmental processes of plants, including the juvenile stages, production of volatile odors, senescence and formation of compounds such as jasmonic acid (Anderson, 1995). This process can be initiated by free radicals or enzymatic activities (Shewfelt and Purvis, 1995). Therefore, the detection of lipid peroxidation is important in research into plant senescence and environmental stress.

Malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, is the important indicator for the process (Auer *et al*., 1995). The Thiobarbituric Acid (TBA) reactive substances assay is an easy and

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quick assay for the assessment of MDA concentration in plants. In this assay, MDA reacts with TBA to form a pink pigment, a condensation product of TBA and MDA in a 2: 1 molar ratio, which has an absorption maximum at 532 nm (Nair and Turner, 1984). In plant tissue, however, certain compounds (anthocyanins and carbohydrates) may interfere with measurements at this wavelength. Furthermore, the presence of barbituric acid impurities in the TBA reagent has been found to produce 1:1:1 TBA/MDA/barbituric acid and 2: 1 barbituric acid/MDA adducts that absorb at 513 and 490 nm (Jardine *et al*., 2002).

The aim of this study is to analyze the applicability of the TBA method for measuring MDA concentrations in various plants. As a further check of the MDA extraction rate, two frequently used solutions, Phosphate Buffer (PB) and Trichloroacetic Acid (TCA), were compared. In golden privet (*Ligustrum* × *vicaryi*) leaves, substances were found that interfered with the measurement of MDA concentration using the TBA method. To separate the interfering substances, fractional extraction and Gas Chromatography-Mass Spectrometry (GC-MS) were used.

2. MATERIALS AND METHODS

2.1. Materials

The functional and the senescent leaves of 12 plants grown at the botanical gardens of the Anhui Agricultural University (eastern China, 32°N) were selected as the materials (**Fig. 1**). The samples were collected, placed immediately into an ice box, weighed, quickly frozen in liquid nitrogen and stored at -80°C.

2.2. Extraction of MDA

Fresh functional or senescent leaves (0.5 g) from at least seven plants were taken and ground in 5 mL extraction solution (separately using 0.05 mol·L^{-1} pH 7.8 PB or 5% TCA as extraction solution), followed by centrifugation at 5000 g for 15 min. The supernatant is the MDA extraction solution, which was stored at 4°C.

2.3. Measurement of MDA

MDA levels were estimated according to the corrected TBA method (Hodges *et al*., 1999). Two milliliters of extraction solution and 3 mL 0.5% TBA including 5% TCA were mixed vigorously. The mixture was heated at 95°C in a constant temperature water bath for 30 min and then cooled in ice to room temperature. After centrifuging at 5000 g_n for 15 min, the supernatant was detected at 450, 532 and 600 nm. The concentration of MDA was determined using the formula C_{MDA} (μmol mL^{-1}) = 6.45 × (D_{532}-D_{600})-0.56×D_{450}, where D_{450}, D_{532} and D_{600} are the absorbencies at 450, 532 and 600 nm, respectively.

2.4. Fractional Extraction for the Interfering Substances

The extraction of MDA was performed using the TBA method. To separate the interfering substances, equal volumes of petroleum ether, ether, ethyl acetate and n-butyl alcohol were successively used as extracting solvents. Products of interfering substances in partial extracts that reacted with TBA were detected and scanned by absorption spectroscopy at 400-700 nm.

2.5. GC-MS Conditions

The ether phase extracted from golden privet and paper mulberry leaves was evaporated to dryness with nitrogen. Then the dried sample was re-dissolved using 2 mL ether for GC-MS analysis. Auto system Shimadzu QP 2010 GC-MS was used to identify the interfering substances. The temperature programming of GC separation was as follows: initial oven temperature was set at 150°C and maintained at that temperature for 2 min, then raised to 210°C at a ramp of 4°C min^{-1} and maintained for 2 min and to 280°C at 5°C min^{-1} and maintained for 7 min. Nitrogen was used as carrier gas with column head pressure at 12.26 kPa in constant pressure mode. The injection volume was 2 μL, the programming split/splitless injection temperature was set at 280°C with a split ratio of 5:1 and the electron capture detector was set at 300°C. The mass spectrometer was operated at 200°C in electron impact mode (70 eV), scanning from m/z 40-600 in 0.3 s with a 0.2 s interval time of the scan; the temperature of the GC-MS interface was 300 °C and the voltage of the photoelectric multiplier tube was 200 V. Mass spectra identification was carried out by comparing with the NIST 107 (National Institute of Standards and Technology, Gaithersburg, USA) and Wiley 6.0 (Wiley, New York, NY, USA) mass spectral libraries.

2.6. Statistical Analysis

Data were expressed as the mean ± SD. The statistical significance of differences between groups was determined with Student's t-test using SPSS software (Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

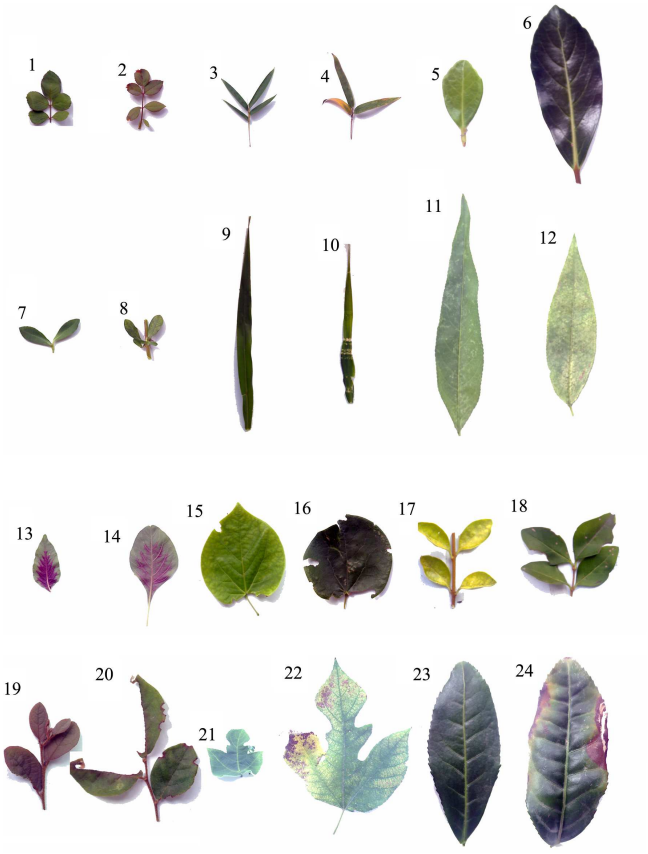


Fig. 1. Leaves of various plants sampled for the analysis of MDA concentrations. Leaves shown are: 1,2: multiflora rose (*Rosa multiflora*); 3,4: bamboo (*Phyllostachys bambusoides*); 5,6: sweet viburnum (*Viburnum odoratissimum*); 7,8: alligator weed (*Alternanthera philoxeroides*); 9,10: green bristlegrass (*Setaria viridis*); 11,12: peach tree (*Prunus persica*); 13,14: amaranth (*Amaranthus gangeticus*); 15,16: Chinese redbud (*Cercis chinensis*); 17,18: golden privet (*Ligustrum* × *vicaryi*); 19,20: red-flowering loropetalum (*Loropetalum chindensevar*); 21,22: paper mulberry (*Broussonetia papyrifera*); 23,24: tea plant (*Camellia sinensis*). For each species, odd numbers indicate functional leaves and even numbers indicate senescent leaves

3. RESULTS

3.1. Effect of MDA Extraction with Two Different Solutions

The extraction solutions used were 0.05 mol L^{-1} pH 7.8 PB and 5% TCA solutions. To compare the extraction efficiency with different solutions, MDA concentrations were measured by the TBA method in 12 different plant species. The MDA concentrations obtained from the two different extraction solutions differed significantly ($p = 0.031$) from each other (**Table 1**). The MDA concentrations with 5% TCA as the extraction solution were on average 32% higher than those with PB as the extraction solution. The solutions extracted with 5% TCA were clearer than those extracted with PB (data not shown). The MDA concentrations in senescent leaves were higher than those in young or mature leaves and the difference between them was significant ($p = 0.025$), except for amaranth and golden privet. The average concentration in senescent leaves was 5.41 μmol g^{-1} fresh weight, whereas in mature leaves it was 3.90 μmol g^{-1} fresh weight. These data indicate that the TBA method with TCA extraction solution was appropriate for analyzing MDA concentrations in most plants tested.

The solution extracted from young leaves of amaranth was redder than the solution from senescent leaves (data not shown). We suggest that anthocyanins in amaranth leaves may be the main interfering substances that influence the accuracy of the TBA method; amaranth leaves have a higher anthocyanin content than leaves of other species. Unexpectedly, the MDA concentrations in golden privet leaves as

measured by this method were negative and there was little red residue in the solutions extracted from these leaves. The solutions of these samples had higher absorption at 600 nm than at 532 nm (**Fig. 2C**).

3.2. Fractional Extraction for Interfering Substances

To further clarify the reason for the apparent negative MDA concentrations detected by the TBA method in golden privet leaves, the interfering substances were separated by successive liquid-liquid extraction. Paper mulberry leaves were used as control material. Petroleum ether, ether, ethyl acetate and *n*-butyl alcohol were used as extraction solvents. Compared with the red products of MDA (solution extracted from paper mulberry) following reaction with TBA, the products of the interfering substances (solution extracted from golden privet) following reaction with TBA appeared dark green (**Fig. 2A and B**). The extraction results show that the concentration of the dark green interfering substance was highest in the ether phase and lowest in the ethyl acetate phase (**Fig. 2A**), whereas the red product of control material (paper mulberry) was highest in the water phase and lowest in the ether phase (**Fig. 2B**).

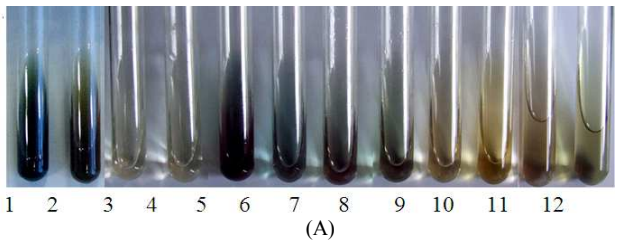
The absorption spectroscopy scanning results showed that the dark green substance had a characteristic absorption peak at 615 nm, whereas the red product of the control had a characteristic peak at 500 nm (**Fig. 2C**). Detection of the interfering substance in different tissues showed that this substance was mainly found in leaves but was almost undetectable in the stems and roots (**Fig. 2D**).

3.3. GC-MS Analysis

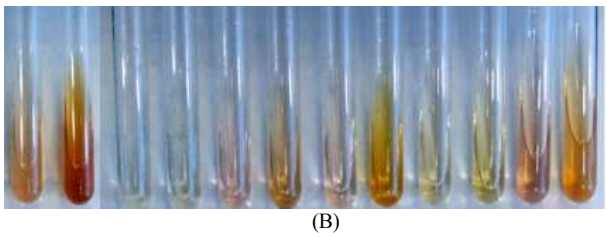
To determine the structure of these MDA-interfering compounds, the ether phases extracted from the golden privet leaves and stems were analyzed by GC-MS (**Fig. 3**). Compared with the gas chromatogram of stems, the gas chromatogram of the ether phase of golden privet leaves showed a few different peaks, such as peaks 8, 9, 10 and 11. The analysis of mass spectrum predicted that peaks 8, 9, 10 and 11 were similar to diethyl hydroxybutanedioate (similarity: 94%), 3, 4-dimethoxybenzoic acid (72%), quinuclidine-2-carboxylic acid, 2, 3-dehydro-3-amino-ethyl ester (63%) and yramine, N-formyl- 2-(4-Hydroxyphenyl) ethylformamide (82%), respectively.

Table 1. Comparison of MDA extraction effect by two different solutions PB means MDA solutions extracted by 0.05 mol L^{-1} pH 7.8 phosphate buffer; TCA means MDA solutions extracted by 5% TCA. The MDA concentration values represent the mean value ± SD. FW, fresh weight

	MDA concentration (μmol g^{-1}FW)			
	PB		TCA	
Plant names	Functional Leave	Senescent leave	Functional leave	Senescent leave
Multiflora rose	4.45±0.99	4.99±1.00	8.10±1.03	8.38±1.21
Bamboo	2.49±0.89	3.61±0.89	5.67±0.96	7.68±0.94
Japan arrowwood	3.44±1.07	1.21±0.77	4.41±1.09	5.44±0.66
Alligator weed	1.22±0.22	1.45±0.19	1.79±0.69	2.15±0.19
Green bristlegass	2.34±0.20	3.35±0.94	2.33±1.00	2.67±0.34
Peach plant	1.09±0.77	2.76±0.97	5.45±0.85	4.13±0.88
Amaranth	1.67±0.91	1.51±0.36	0.83±0.13	0.60±0.59
Chinese redbud	4.70±1.08	4.88±0.88	4.64±0.66	6.12±0.98
Golden Privet	−0.69±0.19	−2.26±0.34	−5.32±0.91	−18.74±1.99
Redflowered Loropetalum	5.63±1.11	9.76±0.93	2.28±0.83	12.9±0.92
Paper Mulberry	2.09±0.35	5.38±0.99	4.09±1.92	4.11±1.00
Tea plant	2.45±0.11	4.07±0.54	3.28±0.97	4.31±0.90



(A)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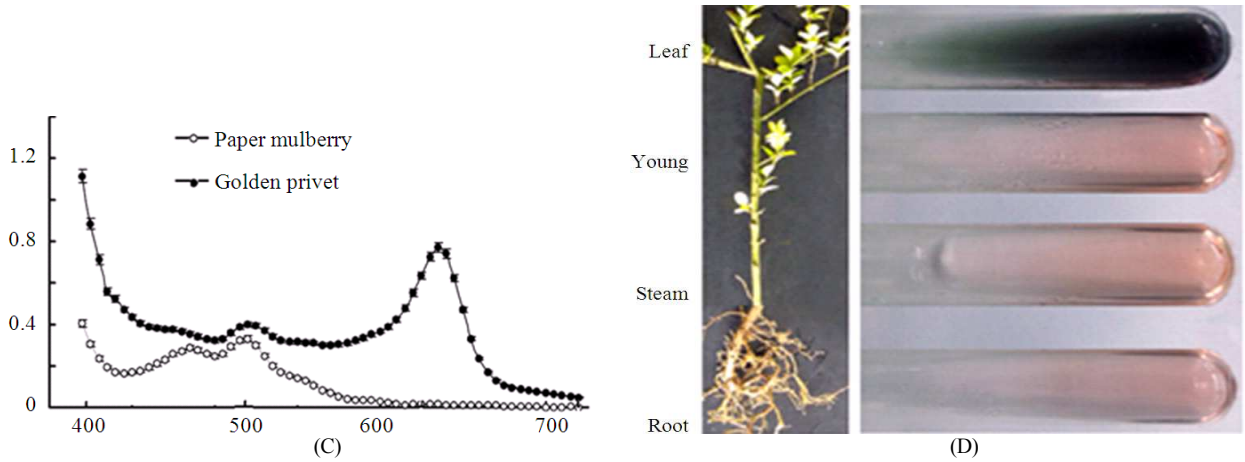
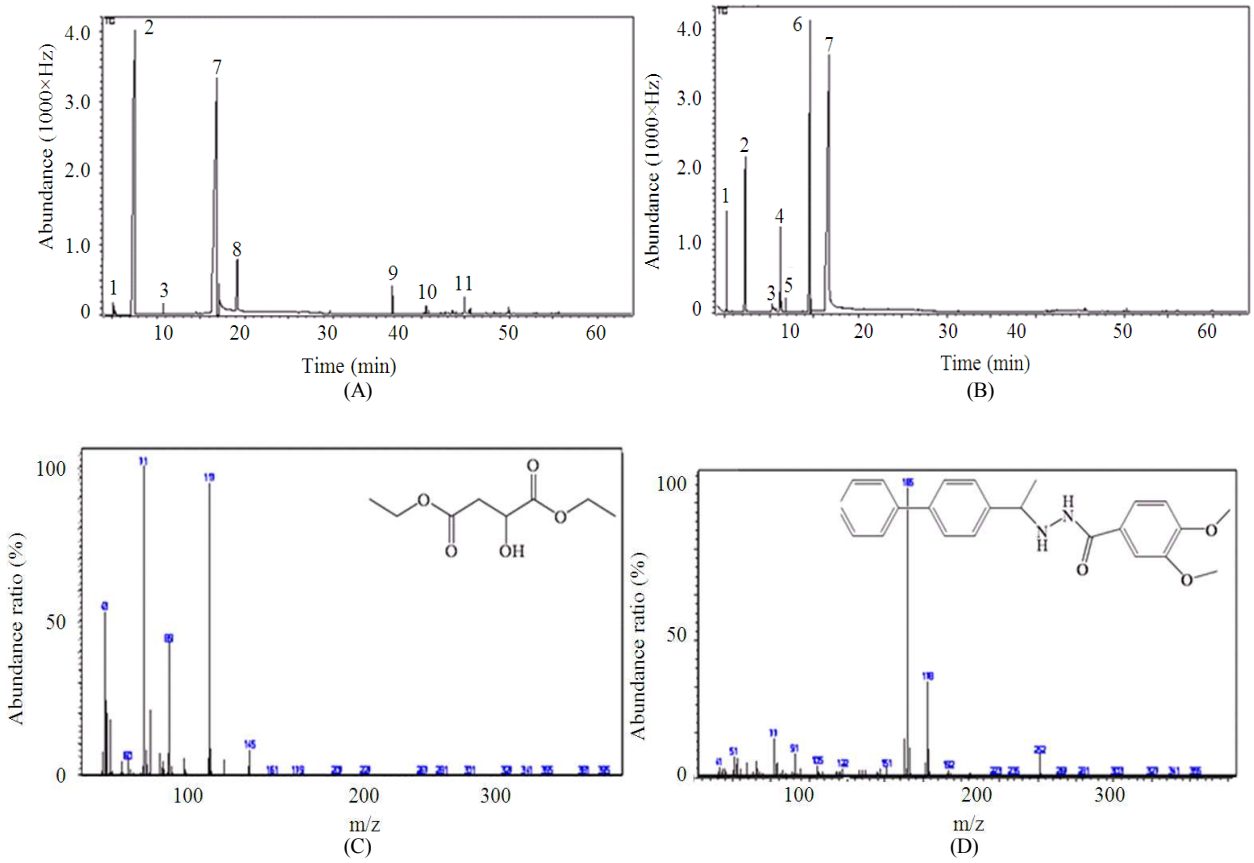


Fig. 2. Separation of the interfering substances by successive liquid-liquid extraction in golden privet and paper mulberry leaves. A: solutions extracted from golden privet; B: solutions extracted from paper mulberry; C: visible light spectra of ether extract solution; D: the accumulation of interfering substances in the different tissues of golden privet. MDA solutions were successively liquid-liquid extracted by: 1,2 5 % TCA; 3,4 petroleum ether; 5,6 ether; 7,8 ethyl acetate; 9,10 *n*-butyl alcohol. 11, 12 show the water phase. For each solution, odd numbers indicate extraction from functional leaves and even numbers indicate extraction from senescent leaves



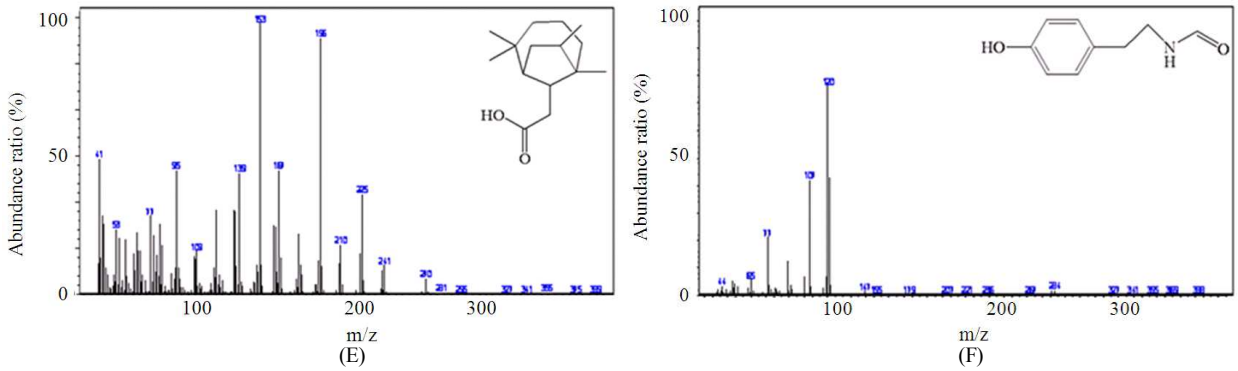


Fig. 3. GC-MS analysis of the MDA-interfering substances in golden privet leaves. Gas chromatograms of ether phase extracted from golden privet leaves (A) and stems (B) and the mass spectra of peaks 8(C), 9(D) and 10 (E) and 11 (F). The mass spectra show that the peaks are most similar to the following compounds: peak 8, diethyl hydroxybutanedioate (similarity: 94%); peak 9, 3,4-dimethoxy-benzoic acid (similarity: 74%); peak 10, quinuclidine-2-carboxylic acid, 2,3-dehydro-3-amino-ethyl ester (similarity: 63%); peak 11, yramine, N-formyl- 2-(4-Hydroxyphenyl) ethylformamide (similarity: 82%)

4. DISCUSSION

The rationale and methodology of the TBA reactive substances assay have been discussed in detail elsewhere (Nair and Turner, 1984; Jardine *et al*., 2002). The method has been rightly criticized for low specificity and artifact formation (Janero and Burghardt, 1988; Knight *et al*., 1988; Davey *et al*., 2005). In recent years, several HPLC-based TBA assays have been developed with increased specificity (Jens, 2001). Despite these facts, the spectrophotometric TBA method remains one of the most useful and commonly used measurements of lipid peroxidation because of its simplicity.

PB is used as a common extraction solution for activity analysis of the enzymes in activated oxygen metabolism (Parussini and Godinot, 1986). It can also be used in lipid peroxidation detection, which is very useful in rare plant studies to save experimental material. The MDA concentrations measured by the TBA method with PB and TCA as extraction solutions differed significantly from each other. The MDA concentrations with 5% TCA as the extraction solution were on average higher than those with PB solution. In agreement with some other studies (Blokhina *et al*., 1999; Du and Bramlage, 1992), the results suggest that lipid peroxidation should be corrected for interfering compounds when measured by the TBA method with TCA as the extraction solution in most experimental plants.

Unexpectedly, an interesting result was detected that in golden privet leaves the MDA concentration was negative when measured by this method. Golden privet, also known as golden vicary privet or golden *Ligustrum*, is a vase-shaped shrub often used for shrub borders and hedging. Leaves of golden privet are golden yellow and lance-shaped. To further clarify the reason for the negative MDA concentrations acquired by the TBA method in golden privet leaves, the interfering substance was separated by successive liquid-liquid extraction. The results indicated that the interfering substances were highly lipid soluble and were present at the highest levels in the leaves and almost undetectable in stems and roots.

The product of MDA following reaction with TBA was measured at 532 nm with 600 nm as the reference wavelength. The latter wavelength is used merely for baseline correction (Jens, 2001). The products of the interfering substances following reaction with TBA had a characteristic absorption peak at 615 nm, which is the reason for the negative MDA concentrations measured by the TBA method in golden privet leaves.

Reaction with TBA yielding colored derivatives is a characteristic of conjugated aldehydes. Futterman and Saslaw (1961) found that vitamin A aldehyde reacts with aqueous TBA to yield a black derivative, which imparts various colors to different organic solvents. Our GC-MS data indicated that a few different substances found in the ether phase of golden privet leaves, in which Peak 11 (similar with N-formyl-2-(4-Hydroxyphenyl) ethylformamide) might be one of aldehyde compounds.

5. CONCLUSION

Comparison of the MDA extraction rate between two commonly used solutions, PB and TCA, suggested that lipid peroxidation should be corrected for interfering

compounds when measured by the TBA method with TCA as extraction solution in most experimental plants. However, the TBA method is not suitable for measuring the MDA concentration in golden privet (*Ligustrum* × *vicaryi*) leaves, because of the existence of interfering substances. The extraction results indicate that the interfering substance was highly lipid soluble and was present at the highest levels in the leaves. Absorption spectroscopy scanning results showed that the products of the interfering substance reacted with TBA had a characteristic absorption peak at 615 nm. The comparison of GC-MS profiles indicated that a few different substances were found in golden privet leaves, in which the Peak 11 might be one of aldehyde compounds, similar with N-formyl-2-(4-Hydroxyphenyl) ethylformamide. Further exploration and a more detailed explanation of the interfering substances will be carried out in a future study.

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