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# A Novel *in Vitro* Whole Plant System for Analysis of Polyphenolics and Their Antioxidant Potential in Cultivars of *Ocimum basilicum*

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**ABSTRACT:** Plants are an important source for medicinal compounds. Chemical screening and selection is critical for identification of compounds of interest. *Ocimum basilicum* (Basil) is a rich source of polyphenolics and exhibits high diversity, therefore bioprospecting of a suitable cultivar is a necessity. This study reports on the development of a true to type novel "in vitro system" and its comparison with a conventional system for screening and selection of cultivars for high total phenolics, individual polyphenolics, and antioxidant content. We have shown for the first time using online acidic potassium permanganate chemiluminescence that extracts from *Ocimum basilicum* showed antioxidant potential. The current study identified the cultivar specific composition of polyphenolics and their antioxidant properties. Further, a distinct relationship between plant morphotype and polyphenolic content was also found. Of the 15 cultivars examined, "Holy Green", "Red Rubin", and "Basil Genovese" were identified as high polyphenolic producing cultivars while "Subja" was determined to be a low producer. The "in vitro system" enabled differentiation of the cultivars in their morphology, polyphenolic content, and antioxidant activity and is a cheap and efficient method for bioprospecting studies.

**KEYWORDS:** *antioxidant, basil, bioprospecting, chicoric acid, chemiluminescence, Ocimum basilicum, rosmarinic acid*

## INTRODUCTION

Phytochemical screening is a preselection step and can be defined as a systematic assessment of plants against biologically important chemical moieties for biotechnological studies or commercial production. Screening for phytochemicals follows two main strategies: direct sampling of plants collected from wild/local markets[1−3] or collection of the seeds or plants from their geographical locations and growing them either in a green house[4,5] or in hydroponic systems.[6,7] Both strategies have disadvantages of loss of elite plant species from natural vegetation and alteration in secondary metabolite profile due to variable growth conditions,[8,9] thereby raising the need to develop a system that is true to type, is effective, and enables reproducible plant screening.

Polyphenolics having pharmaceutical value are a major class of secondary metabolites found in the family Lamiaceae. This family includes herbs such as rosemary, salvia, melissa, thyme, and basil, the aerial parts of which are reported to be rich sources of polyphenolics, for example, rosmarinic, caffeic, urosolic, chicoric, and salvianolic acids.[10−12] These herbs have been extensively studied for their polyphenolic composition[5] and content both in conventional as well as in in vitro systems (tissue culture and hairy root culture).[13] *Ocimum basilicum* has been less investigated in vitro (tissue culture and hairy root culture) in comparison to other lamiaceae members, making it a potential lead plant for polyphenolic studies.

*Ocimum basilicum* is referred to as "king of the herbs" and was sourced originally from tropical and subtropical Asia for its medicinal, culinary, and ornamental properties. This herb is grown economically worldwide for its use in pharmaceuticals, cosmetics, and in the food industry.[14] Biologically, it shows antibacterial, antithrombotic, antioxidant, anti-inflammatory, and antihypertensive[15−20] activities. Breeding and hybridization techniques are practiced for cultivation of *Ocimum basilicum*[14] and are the major reason for its high genetic diversity of 65−150 species[14] with different morphological traits,[18,21] volatile oil composition,[15,22] and phenolic content.[4,18]

Rosmarinic (RA), chicoric (ChA), and caffeic (CA) acids are the main polyphenolics found in *Ocimum basilicum*.[2,4,18] Of these polyphenolics, rosmarinic acid is the most abundantly found polyphenolic acid. Rosmarinic acid is reported to have antioxidant, anti-inflammatory, neuroprotective, hypoglycemic, and antiproliferative properties[23−27] and is found in a number of commercial products such as Neurex, Persen, and Aquarox.[28]

Chicoric acid (dicaffeoyltartaric acid), found as a major metabolite in echinacea and chicory,[2,3,29] is also known to have antioxidant, anti-inflammatory, antiviral, and hypoglycemic activity.[2,3,30,31] Extracts of *Echinacea*, a rich source of chicoric acid, are widely used as dietary supplement, showing the great market potential of natural products and the need for identification of new plant sources. Recently, *Ocimum basilicum* has been identified to be an alternative and less expensive source of chicoric acid.[2,3,29]

There are studies on *Ocimum basilicum* that have reported the collection, screening, and identification of the cultivars that have high yields of rosmarinic, chicoric, caffeic, and caftaric acids and high antioxidant potential.[2,4,18,19,32] Effects of light,[33]

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nutrients,[9] and mycorrhization[2,34] on polyphenolics profile of basil have also been studied. These studies have been carried out using conventional pot (CP system) grown plants in the greenhouse[2,4,18,19,32,34] or in hydroponic systems.[6,7] Several studies also report collection of plants from wild or market bought samples for polyphenolics study.[2,3] For the screening of large and diverse collection of cultivars of *Ocimum basilicum*, use of a conventional pot system as a screening platform is a large, cumbersome, and voluminous technique requiring high cost and physical operations. Therefore, a system which can be managed in less space and observed throughout with visibility of the root system, as is not the case in a conventional pot system, is needed for screening studies of *Ocimum basilicum*.

For the selection of basil cultivars to be used for medicinal and food applications, determination of antioxidant potential also plays an important role. Conventionally, determination of antioxidant potential utilizes spectrophotometric assays based on 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) and 2,2′-azinobis(3-ethylbenzothioazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) chromogens.[35] To increase the efficiency of detection these assays have been coupled with a HPLC postcolumn separation where the antioxidant potential of individual constituents can be assessed.[35−37] Postcolumn detection using the conventional chromogens has the disadvantage of long processing times, which results in poor resolution of the resulting chromatogram.[36] Chemiluminescence signals produced by the use of an acidic potassium permanganate assay overcome this issue due to the fast nature of the light producing reaction involved, and this technology has been used for polyphenolics analysis in many studies.[35−42]

Assays based on chemiluminescence offer selectivity to antioxidants, high sensitivity, ease of chemical preparation, long shelf life of the reagent, fast analysis time, and simple instrumentation. A positive correlation between the acidic potassium permanganate and the traditional DPPH assay has been also observed, making this technology an ideal replacement for the cumbersome online DPPH assay.[35] Further to this a direct correlation between acidic potassium permanganate chemiluminescnce signal and bioactive potential in muscle cell culture has been determined.[39] The distinct advantages of chemiluminescence based assays enable them to be used as a test for estimation of total antioxidant potential and for the assessment of individual chromatographically separated components.

This study developed a new "in vitro" true to type (as offspring plants raised are phenotypically and metabolically similar to the parent plant) system for plant growth in a controlled environment that allowed morphotyping, screening, and selection of the high yielding cultivars of *Ocimum basilicum* for three marker compounds rosmarinic, chicoric, and caffeic acids (referred to as polyphenolics in this study). Acidic potassium permanganate chemiluminescence detection was used to determine the antioxidant activity for whole extracts and key compounds of interest. We believe that the system has distinct advantages of uniformity, high reproducibility, and efficiency over the CP system approaches currently used in plant science for screening and selection studies.

## MATERIALS AND METHODS

**Plant Material.** Seeds of 15 different *Ocimum basilicum* cultivars from five different countries (India, Germany, Spain, United States of America, and Australia) were used in this study for representation of morphological variability and chemical diversity. Seeds were purchased from seed suppliers and research institutes. The seeds of each cultivar were tested for viability by first washing with 0.1% (v/v) Tween 20 for 3 min, then with tap water to remove excess detergent, followed by surface sterilization with 0.01% $HgCl_2$ two times for 2 min followed by washing with sterile distilled water three times. The surface sterilized seeds were placed on Murashige and Skoog (MS) medium within 90 mm diameter Petri plates (25 seeds in each plate). The plates were then incubated in the dark in a plant growth room at 25 ± 2 °C at 60% relative humidity. After 10−20 d the percentage germination for each seed lot was determined and seed lots were characterized on the basis of germination percentage as high (70−100%), medium (31−69%), low (21−30%), and very low or nil (0−20%).

**Chemicals.** Mercuric chloride ($HgCl_2$) and Tween 20 used for seed sterilization and standards of gallic acid, rosmarinic acid, chicoric acid, and caffeic acid were obtained from Sigma-Aldrich (Castle Hill, Australia), and Murashige and Skoog medium was prepared following the method of Murashige and Skoog.[43]

Ethanol and 85% *o*-phosphoric acid (AR grade) were obtained from Merck (Kilsyth, Australia). HPLC grade methanol was obtained from BDH Chemicals (Poole, England). Millipore Millex-HN (syringe filters; 0.45 μm) that were used for filtration of samples were obtained from Merck (Darmstadt, Germany).

For the chemiluminescence assays, potassium permanganate was obtained from Chem Supply (Gillman, Australia), sodium polyphosphate was obtained from Sigma-Aldrich (Castle Hill, Australia), and analytical grade sulfuric acid was from Merck (Kilsyth, Australia).

MS medium was prepared by dissolving $KNO_3$ (0.37 M), $NH_4NO_3$ (0.41 M), $MgSO_4{\cdot}7H_2O$ (0.06 M), $KH_2PO_4$ (0.02 M), $CaCl_2{\cdot}2H_2O$ (0.08 M), $MnSO_4{\cdot}4H_2O$ (0.01 M), $ZnSO_4{\cdot}4H_2O$ (0.04 M), $H_3BO_3$ (0.01 M), KI ($4.81 \times 10^{-4}$ M), $Na_2MoO_4$ ($1.21 \times 10^{-4}$ M), $CuSO_4{\cdot}5H_2O$ ($1.57 \times 10^{-5}$ M), $CoCl_2{\cdot}6H_2O$ ($1.93 \times 10^{-3}$ M), $Na_2EDTA$ (0.01 M), $FeSO_4{\cdot}7H_2O$ (0.01 M) (all from Merck), glycine (0.002 M), nicotinic acid ($4.06 \times 10^{-4}$ M), pyridoxine hydrochloride ($2.95 \times 10^{-4}$ M), thiamine hydrochloride ($2.96 \times 10^{-5}$ M), and myoinositol (0.05 M) in 1 L of distilled water with 2.5% phytagel (all from Sigma-Aldrich, St. Louis, MO), and the pH was maintained at 5.8. The prepared medium was then autoclaved at 121 °C, 15 psi for 15−20 min.

***In Vitro* System.** An *in vitro* [true to type] whole plant system was developed. A 90 mm diameter Petri dish containing MS medium was used as the plant growth substrate. To enable plant growth from the sterile substrate, a hole was made in the lid of the dish. A germinated seedling at the two leaf stage was transferred using forceps and inserted leaf first through the hole in the lid of the Petri plate that had been removed from the base. The lid containing the inserted seedling was then gently placed back on the base which contained the MS medium so that the root was in contact with the medium. After successful transfer any space around the stem was sealed with sterile silicone grease. The Petri dish containing a seedling was placed on a flat bench, and then the plate was covered with a black paper sheet that had a hole in the center from which the stem emerged (Figure 1A). The black sheet was used to minimize light incidence on the developing root system. The same steps were repeated for each of 15 cultivars that were arranged in a completely randomized block design with three replicates for each. The plants were grown under controlled conditions at 25 ± 2 °C under cool white fluorescent lights (Thermoline Scientific, Wetherill Park, NSW, Australia) that had a photosynthetic photon flux density of 100−120 $\mu M\ m^{-2}\ s^{-1}$ for a photoperiod of 16 h light and 8 h dark with 60% humidity.

**Conventional Plant System (CP System).** For comparison with the in vitro whole plant growth system, a conventional pot experimental system was also set up. For this purpose, seeds were surface sterilized using the procedure previously described and then sown in trays in sterilized commercially obtained soil (Loess soil) mixed with absorbent granules (TERRA-GREEN, Greenscape Aeration Company, Atascadero, CA) and kept in the dark. After germination one seedling at the two leaf stage was transferred to a 5 L black plastic pot containing the soil:granule mix. The experiment was set up with all cultivars with three replicates each in a completely randomized block design in a greenhouse at 25−28 °C. The plants

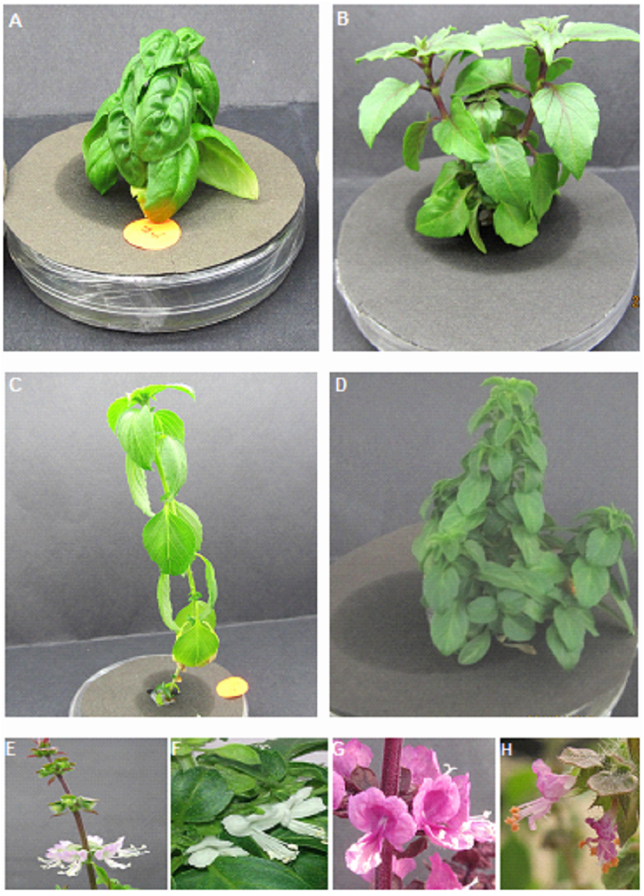


**Figure 1.** *Ocimum basilicum* cultivars grown in the *in vitro* setup developed in a 90 mm Petri dish. Four different morphotypes were observed in the study: (A) large (lettuce) leaf and robust type; (B) purple colored type; (C) tall and slender type; (D) dwarf leaf type. There were four types of flower found in the study: (E) light purple; (F) white flower; (G) dark purple flower; and (H) purple flower.

were watered to pot capacity on a daily basis and fertilized fortnightly with Hoagland solution.[44]

**Plant Harvesting, Morphotyping, and Lyophilization.** Plants were grown to maturity in both systems and examined for variation in aerial morphology. The morphology of each cultivar was classified according to the traits used by Carović-Stanko et al.,[21] who used the morphological traits developed by the International Union for the Protection of New Varieties of Plants for *Ocimum basilicum L* (UPOV 2003).[45] Plant height and internodal distance was measured on harvested plants from the base of the stem to the apical bud using a standard mm scale. After harvest, samples were lyophilized (Labconco lyophilizer, Kansas City, MO, United States of America) at −94.3 °C and 141 kPa for 48 h, and then the lyophilized weight was recorded (data not shown).

**Extraction for Total Phenolics, Individual Polyphenolics, and Antioxidant Potential Analysis.** For total phenolics, individual polyphenolics (rosmarinic chicoric and caffeic acids), total antioxidant, and individual antioxidant potential studies using postcolumn chemiluminescence assay, 50 mg of lyophilized plant material (aerial and root) was homogenized separately in 500 μL of 60% ethanol. To the homogenized mixture was added 14.5 mL of 60% ethanol, and the mixture was sonicated at 25 °C using an ultrasonicator (Branson Ultrasonics, Danbury, CT, United States of America) for 10 min. The sonicated extract was then centrifuged at 10,000 rpm for 5 min, the supernatant was collected in a 25 mL volumetric flask, and the leftover residue was re-extracted in 10 mL of 60% ethanol for 5 min and then centrifuged as previously. The supernatant was then pooled in volumetric flasks that contained the first fraction, and the final volume was made up to 25 mL with 60% ethanol. The extracts were then filtered and stored in the dark at −80 °C in HPLC vials for total phenolics, individual polyphenolics, and chemiluminescence studies.

**Determination of Total Phenolics.** For the determination of total phenolics, a modified Folin−Ciocalteau colorimetric assay[46] was used. Briefly, to 100 μL of the ethanolic extract were added 400 μL of distilled water, 250 μL of Folin−Ciocalteau reagent, and 1.25 mL of 2.1% aqueous sodium carbonate, the mixture was incubated in the dark for 30 min, and the absorbance of the resulting mixture was taken at 735 nm using a microplate reader (SPECTRAmax 340 PC$^{384}$ Microplate Spectrophotometer, Sunnyvale, CA, United States of America) against the same mixture without sample. The total phenolic concentration was quantified from a standard curve prepared for gallic acid within the range of 20−100 mg/L, and the final concentration of total phenolics in a sample is reported as gallic acid equilvalents (GAE mg/g DW).

**Determination of Rosmarinic, Chicoric, and Caffeic Acid Contents by HPLC Analysis.** Chromatographic analysis was carried out using HPLC (Agilent Technologies 1200 series liquid chromatography system), equipped with a quaternary pump, solvent degasser system, autosampler, and diode array detector (Agilent Technologies, Victoria, Australia). Inbuilt software (Hewlett-Packard Chemstation, Agilent Technologies) was used to control the HPLC pump and acquire data from the diode array, UV−vis absorbance, and chemiluminescence detectors. Separations were performed on an Apollo TM C 18 (150 mm × 4.60 mm × 5 μm particle diameter) column.

For separation of individual polyphenolics the mobile phase used was HPLC grade water + 0.1% OPA (mobile phase A) and methanol + 0.1% OPA (mobile phase B). A gradient program was developed for RA quantitation: 0−2 min isocratic 0% B, 2−5 min linear gradient to 40% B, 5−10 min a linear gradient to 50% B, 10−18 min isocratically maintained at 50% B, 18−23 min a decreasing gradient from 50% to 40%, and finally 23−25 min 0% B for column washing. The flow rate of the mobile phase was 1.0 mL/min, and the wavelength used for detection of all three acids was 280 nm with an injection volume of 20 μL. Unknown samples were identified by comparison of the retention times with those of commercial standard. For the sensitivity study of RA, ChA, and CA, 20 standard samples were prepared between $1 \times 10^{-3}$ M and $1 \times 10^{-12}$ M. Quantification of unknown samples was determined by comparison of integrated peak area for each sample with a standard calibration curve of rosmarinic, chicoric, and caffeic acids.

**Determination of Total Antioxidant Potential.** Total antioxidant potential was determined using acidic potassium permanganate chemiluminescence detection coupled to an Agilent 1200 HPLC system. The HPLC system (without a column in place) was coupled with a Minipuls 3 peristaltic pump, bridged PVC tubing, and custom built luminometer (which functions similarly to a conventional flow injection analysis system). Both the in vitro and CP system whole plant samples were diluted 100 times using deionized water before injection of 50 μL of the sample at flow rate of 3 mL min$^{-1}$ and merging with acidic potassium permanganate ($1 \times 10^{-3}$ M), and the peak area was recorded. The acidic potassium permanganate was prepared by dissolution of potassium permanganate in 1% sodium polyphosphate solution adjusted to pH 2 with sulfuric acid.

**Determination of Individual Antioxidant Potential by Chemiluminescence Assay.** In vitro grown plant sample extracts were subjected to postcolumn acidic potassium permanganate assay to assess the contribution of individual polyphenolic compounds. All polyphenolic standards ($1 \times 10^{-3}$ M to $1 \times 10^{-12}$ M) were prepared in 100% analytical grade methanol and appropriately diluted with the same solvent. Postcolumn acidic potassium permanganate assay was performed using the system previously described.[36] Postcolumn acidic potassium permanganate chemiluminescence was generated using an in house built manifold. The reagent, propelled at a flow rate of 3 mL min$^{-1}$ using a Gilson Minipuls 3 peristaltic pump (John Morris Scientific, Balwyn, Australia) with bridged PVC tubing (DKSH), merged with the HPLC eluate at a T-piece, and the light emitted from the reacting mixture was detected with a custom built flow-through

luminometer, which consisted of a coiled flow cell comprising 80 cm × 0.8 mm i.d., transparent PTFE-PFA tubing (DKSH), mounted flush against the window of an Electron Tubes photomultiplier tube (model 9828SB, ETP) set at a constant voltage of 900 V from a stable power supply (PM20D, ETP) via a voltage divider (C611, ETP). The flow cell photomultiplier tube and voltage divider were encased in a padded light-tight housing, and a Hewlett-Packard analogue to digital interface box (Agilent Technologies) was used to convert the signal from the chemiluminescence detector.

**Statistical Analysis.** All data presented in this study is expressed in terms of mean ± SEM. Raw data was analyzed using a commercial statistical package (GraphPad Prism 6). One-way analysis of variance with a Tukey HSD test of significance at $p < 0.05$ was used to determine the effect of cultivar on the quantified morphological traits, total phenolic content, individual polyphenolics content, and antioxidant potential for both systems.

Two-way ANOVA analysis using Sidak's multiple comparison test was used to examine the difference between the two growth systems for total phenolics, the individual polyphenolics, and total antioxidant potential. Correlation between total polyphenolics, total polyphenolic antioxidant potential, and rosmarinic acid antioxidant potential was tested using Pearson test at a significance level of 0.05.

Hierarchical cluster analysis was performed using IBM SPSS Statistics 22 to determine if there was a relationship between the cultivars grown in the in vitro system and their polyphenolic constituents. Data used was standardized using $Z$ score and between group linkages were analyzed using squared Euclidean distance and represented in the form of a dendrogram.

## RESULTS AND DISCUSSION

**Germination Rates of Seeds from Selected Cultivars.** Seeds were collected from different seed suppliers and research institutes from five countries. Of the 29 cultivars collected 14 showed no or very low germination and were not used further in this study. Thus, 15 cultivars were used for the analyses (Table 1).

**Morphotyping of Cultivars.** There are 27 traits mentioned in the UPOV guidelines for *Ocimum basilicum*.[45] They can be classified into four major groups, namely, plant type (habitat; height; density), stem type (color; pubescence; number of flowering shoots), leaf type (blade length; pubescence; color; margin; glossiness), and flower type (corolla color; bract hairiness; color of style). Collected cultivars were assessed against these major qualitative and quantitative traits (Table 2). The collected cultivars were broadly classified into large (lettuce) leaf and robust type, dwarf leaf type, purple colored type, or tall and slender type (Figure 1). All the cultivars grew successfully in both the in vitro and CP systems. Plants were healthy at maturity and progressed to flowering (approximately 90 days for the CP system grown plants). Flowering in the in vitro system was either accelerated, with some cultivars flowering at 60 days (for example B2, B8, and B10), or delayed, with flowering after 90 days. Plant height in the CP system was generally much greater than that in the in vitro system, with some cultivars grown in the CP system 20 times the height of those in the in vitro system (for example, B1 3.37 ± 0.44 cm in vitro and 74.83 ± 3.17 cm in the CP system). Height difference was reflected in the greater number of nodes, branches, and internodal distances found for the CP system grown plants. Traits shown in Table 2 align with other studies of *Ocimum basilicum*[18] where morphological diversity is reported.

**Table 1. Seed Collection and Screening of the *Ocimum basilicum* Cultivars for the Study Based on Their Germination Percentage[a]**

| code | cultivar | country |
|---|---|---|
| B1 | Cim Saumya | India |
| B2 | Turkmaniya Basil | India |
| B3 | Subja | India |
| B4 | Ban Tulsi | India |
| B5 | Basilicum Breitblattriges | Germany |
| B6 | Basil Genovese | India |
| B7 | Basil Genovese | Spain |
| B8 | Basil Minimum | Spain |
| B9 | Organic Cinnamon | Australia |
| B10 | Organic Thai Basil | Australia |
| B11 | Basil Stella | Australia |
| B12 | Holey Green | Australia |
| B13 | Red Rubin | Australia |
| B14 | Basil Genovese America | United States of America |
| B15 | Sweet Basil | United States of America |

[a]29 cultivars of *Ocimum basilicum* were collected for the study. B16−B29 (not shown in this table) showed no or very low germination (0−20%) and were not used in the study. B7, B8, B12, and B13 showed low germination percentage (21−30%); B1, B2, B3, B5, and B6 showed medium germination percentage (31−69%); and B3, B9, B14, and B15 showed high germination percentage (70−100%).

**Standardization of a Method for Extraction of Rosmarinic Acid.** Lyophilization of plant samples was determined, through preliminary analysis, to yield the highest concentration of polyphenolics (data not shown). This result concurs with that found previously[3] in a comparison of basil that was lyophilized with fresh, quick frozen or blanched and frozen samples. The removal of water concentrates polyphenolics in the dried plant samples. For *Ocimum basilicum,* RA is the major metabolite of interest, thus our extraction method was developed for its isolation. We tested a number of extraction protocols using ethanol, homogenization, and two forms of sonication (water bath assisted or ultrasonication) to optimize yields of RA from lyophilized samples. The highest yields of RA, 33.58 ± 0.95 mg/g DW and 31.45 ± 0.68 mg/g DW (Figure 2), were found when 80% v/v ethanol in water or 60% v/v ethanol in water respectively was used as the extraction solvent in combination with water bath sonication. Routinely, 60% v/v ethanol in water was used with the sonication processing of all samples. This protocol was found to be time efficient, required minimal resources, and was easy to perform.

**Comparison of the in Vitro and CP System.** *Total Phenolics.* The Folin−Ciocalteau phenolic assay was used to quantitate the total phenolic content of samples derived from the in vitro and CP growth systems for whole plant and was expressed in terms of gallic acid equivalents per gram (Figure 3). There was a significant difference ($p < 0.05$) found in total phenolic content between the two growth systems. It was also found that cultivars grown in the CP system had higher total phenolic contents; for example, the highest phenolic content was found in three of the cultivars, B12 (156.15 ± 6.19 mg/g GAE DW), B13 (127.41 ± 3.11 mg/g GAE DW), and B14 (126.41 ± 0.72 mg/g GAE DW), while the lowest was found for B10 (73.86 ± 1.29 mg/g GAE DW). The in vitro system showed a similar trend of phenolic content among cultivars, and the highest was found in B12 (69.69 ± 2.83 mg/g GAE DW), B13 (67.79 ± 0.82 mg/g GAE DW), and B7 (65.71 ± 2.14 mg/g GAE DW) while the significantly lowest level was found in B15 (41.78 ± 0.99 mg/g GAE DW). The highest

**Table 2. Morphotyping of the 15 Cultivars of *Ocimum basilicum* Grown in the *in Vitro* System and the CP System[a]**

| | in vitro system | | | | CP system | | | |
|---|---|---|---|---|---|---|---|---|
| code | plant height (cm) | no. of nodes | no. of branches | internodal distance (cm) | plant height (cm) | no. of nodes | no. of branches | internodal distance (cm) |
| B1 | 3.37 ± 0.44 c | 6.33 ± 0.33 cde | 12.71 ± 0.66 abc | 0.33 ± 0.08 c | 74.83 ± 3.17 bcde | 12.00 ± 0.58 c | 24.00 ± 1.15 c | 8.77 ± 0.27 ab |
| B2 | 4.31 ± 0.15 bc | 9.00 ± 0.57 bc | 18.00 ± 1.15 abc | 0.50 ± 0.05 c | 75.00 ± 0.58 bcde | 11.33 ± 0.33 cd | 22.67 ± 0.67 cd | 9.17 ± 0.18 ab |
| B3 | 5.53 ± 0.38 abc | 9.00 ± 0 c | 18.00 ± 0 abc | 0.31 ± 0.05 c | 101.70 ± 6.98 ab | 14.67 ± 1.86 bc | 29.33 ± 3.71 bc | 8.60 ± 1.10 ab |
| B4 | 5.12 ± 0.28 abc | 9.00 ± 0 bc | 18.00 ± 0 abc | 0.43 ± 0.03 c | 69.36 ± 5.83 de | 11.33 ± 0.33 cd | 22.67 ± 0.67 cd | 8.23 ± 0.32 ab |
| B5 | 4.93 ± 0.37 abc | 7.67 ± 0.33 bcde | 15.32 ± 0.66 abc | 0.41 ± 0.05 c | 88.83 ± 2.59 abcd | 19.33 ± 0.88 ab | 38.67 ± 1.76 ab | 6.13 ± 0.19 bcd |
| B6 | 4.51 ± 0.37 abc | 8.33 ± 0.33 bcd | 16.71 ± 0.66 abc | 0.42 ± 0.05 c | 79.60 ± 7.41 abcd | 14.33 ± 0.88 bc | 28.67 ± 1.76 bc | 6.87 ± 0.70 bc |
| B7 | 3.47 ± 0.20 c | 7.67 ± 0.33 bcde | 15.31 ± 0.74 ab | 0.93 ± 0.08 abc | 85.20 ± 7.05 abcd | 16.00 ± 0.58 abc | 32.00 ± 1.15 abc | 6.40 ± 0.56 bc |
| B8 | 8.13 ± 1.41 abc | 7.67 ± 0.37 bcde | 15.33 ± 0.66 a | 0.93 ± 0.08 abc | 51.86 ± 4.68 ef | 21.33 ± 2.96 a | 42.67 ± 5.93 a | 2.20 ± 0.36 d |
| B9 | 6.50 ± 0.45 abc | 12.71 ± 1.20 a | 25.31 ± 2.41 abc | 1.57 ± 0.12 a | 94.30 ± 1.64 abcd | 15.67 ± 1.45 abc | 31.33 ± 2.91 abc | 11.83 ± 0.83 a |
| B10 | 9.60 ± 2.79 ab | 9.33 ± 0.88 b | 18.72 ± 1.76 bc | 0.81 ± 0.50 abc | 26.86 ± 7.95 f | 5.33 ± 0.88 d | 10.67 ± 1.76 d | 3.73 ± 0.89 cd |
| B11 | 9.73 ± 2.15 a | 6.67 ± 0.66 bcde | 13.31 ± 1.33 bc | 1.00 ± 0.11 abc | 72.16 ± 9.31 cde | 14.00 ± 1.15 bc | 27.33 ± 2.91 bc | 6.93 ± 0.23 bc |
| B12 | 5.93 ± 0.44 abc | 5.67 ± 0.33 de | 11.33 ± 0.66 c | 1.41 ± 0.21 ab | 78.70 ± 4.31 abcde | 11.33 ± 0.33 cd | 22.67 ± 0.67 cd | 9.13 ± 1.95 ab |
| B13 | 6.41 ± 0.37 abc | 6.67 ± 0.33 bcde | 13.31 ± 0.66 abc | 0.86 ± 0.12 abc | 92.66 ± 1.09 abcd | 17.33 ± 0.88 abc | 34.67 ± 1.76 abc | 6.07 ± 0.28 bcd |
| B14 | 5.17 ± 0.68 abc | 8.00 ± 0.33 bcde | 15.32 ± 0.66 bc | 0.61 ± 0.11 bc | 105.23 ± 3.47 a | 16.33 ± 0.88 abc | 32.67 ± 1.76 abc | 8.13 ± 0.47 ab |
| B15 | 3.43 ± 0.33 c | 5.00 ± 0.57 e | 10.00 ± 1.15 c | 0.66 ± 0.17 bc | 99.00 ± 3.06 abc | 17.00 ± 0.58 abc | 30.67 ± 3.53 abc | 7.57 ± 1.03 bc |

[a]Data represented as mean ± SEM of each of three replicates ($n = 3$). Different letters indicate significant differences ($p \leq 0.05$) according to Tukey's HSD. Plant habitat: All erect type except for B8, showing intermediate habitat. Plant density: Varies from loose (B1, B2, B4) to medium (B9, B10, B15) to dense (B3, B5, B6, B7, B8, B12, B13, B14). Stem type: Stem color in all cultivars was green except for B9 and B13, and hairs were present in all. Leaf type: Variants in leaf morphology including ovate/dark green/less hairy/serrated/flat (B1, B2, B4), ovate/green/hairy/serrated/undulate (B9), lanceolate/purple/serrated/flat (B13), broad ovate/green/hairy/serrated/undulate (B12), small ovate/dark green/serrated/flat/less hairy (B8), broad ovate/green/serrated/undulate/flat/glossy (B3, B5, B6, B7, B11, B14, B15) and lanceolate/green/serrated/less hairy (B15) were observed. Flower type: Four types of flower colors, light purple (B1, B2, B4, B10), white (B3, B5, B6, B7, B8, B11, B14, B15), dark purple (B9, B13), and purple type (B12), were found in this study.



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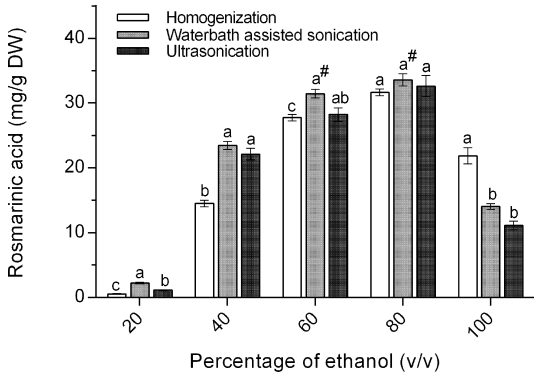


**Figure 2.** Comparison between homogenization, water bath sonication and ultrasonication at five different percentages of ethanol for standardization, and selection of extraction methodology for rosmarinic acid. Data are presented as the mean ± SEM of each of three replicates ($n$ = 3). Different letters indicate significant differences ($p \leq 0.05$) between methods, within percentage of ethanol according to Tukey's HSD. # shows no significant difference ($p \leq 0.05$) between 60% ethanol and 80% ethanol with water bath sonication.

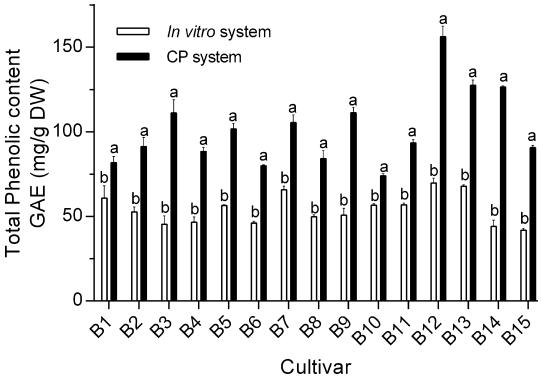


**Figure 3.** Total phenolic content for 15 *Ocimum basilicum* cultivars grown in the *in vitro* system and the CP system. Total phenolic content is reported in gallic acid equivalents, GAE (mg/g DW). Data are presented as mean ± SEM of each of three replicates ($n$ = 3) for the whole plant. Different letters indicate significant differences ($p \leq 0.05$) between the two growth systems, within cultivars according to Sidak's multiple comparison test.

phenolic content was thus found in B12 and B13 for both growth systems. This analysis clearly shows the effect of cultivar on the total phenolic content which correlates well with reports for this species and other studies.[4,47]

Our values for CP system grown samples are of the same magnitude as those reported by Jaysinghe et al.[19] for different fractions used by them. Total phenolic content is dependent on age and state of plant material (dry/fresh), and the contents found in mature plants in our study were much higher than those found for four week old plants (17.58 mg/g GAE DW) in the study reported by Kwee and Niemeyer[4] and Lee and Scagel.[2] Even though determination of total phenolics in plant cultivar samples is a useful comparative measure and may correlate well with antioxidant activity, it provides little indication of the responsible compounds.

*Individual Polyphenolic Content.* HPLC was used to determine the concentrations of RA, ChA, and CA in both the aerial parts and roots of the different cultivars of *Ocimum basilicum* grown in the in vitro and CP systems. A characteristic chromatogram (Figure 4) of extracts from the aerial part and

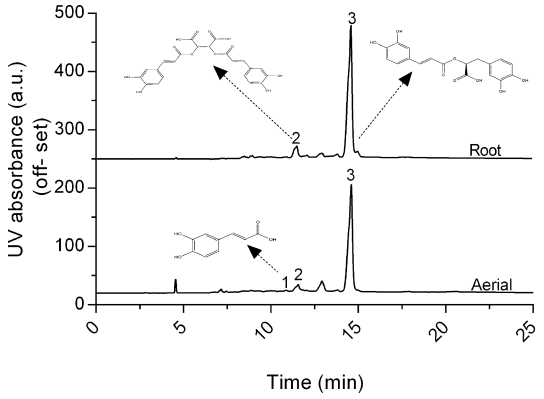


**Figure 4.** Typical HPLC chromatogram for the aerial and root samples of *Ocimum basilicum* using UV absorbance detection (280 nm). Peak 1: caffeic acid (10.6 min). Peak 2: chicoric acid (11.6 min). Peak 3: rosmarinic acid (14.5 min).

roots of B13 showed distinct peaks for RA (14.5 min), ChA (11.6 min), and CA (10.9 min). Analytical figures of merit for three polyphenolics are shown in Table 3. As reported by

**Table 3. Analytical Figures of Merit for Polyphenolics Found in This Study**

| | UV detection | | chemiluminescence | |
|---|---|---|---|---|
| polyphenolic | $R^2$ | LOD ($\mu$M) | $R^2$ | LOD ($\mu$M) |
| rosmarinic acid | 0.9988 | 0.5 | 0.9984 | 0.75 |
| chicoric acid | 0.9900 | 0.2 | 0.9870 | 0.1 |
| caffeic acid | 0.9922 | 10 | 0.9985 | 5 |

earlier studies, RA was found as the major polyphenolic in the ethanolic extract of these cultivars. The highest and lowest RA producing cultivars for whole plants were the same for both growth systems. No significant difference ($p < 0.05$) in RA content of whole plants was found for 11 out of the 15 cultivars grown in both systems. For four of the cultivars there was a significantly higher level of RA in CP system grown plants compared with those grown in vitro. B13, B12, and B7 were found to be high producing cultivars in both systems whereas B3, B5, and B11 emerged as low producing cultivars in both systems. RA content found in whole plants grown in the in vitro system ranged from as low as 24.20 ± 2.31 mg/g DW in B3 to 89.44 ± 1.49 mg/g DW in B13. B13 (89.44 ± 1.49 mg/g DW), B12 (78.00 ± 2.69 mg/g DW), and B7 (52.09 ± 6.38 mg/g DW) were the highest producing cultivars in the in vitro system while B3 (24.20 ± 2.31 mg/g DW), B14 (26.39 ± 0.82 mg/g DW), and B5 (28.46 ± 1.27 mg/g DW) were identified as the lowest producing cultivars (Figure 5A).

As shown in Tables 4 and 5 the aerial parts are found to be the major reserve of RA in both the in vitro system (10/15 cultivars) and the CP system (11/15 cultivars). The RA content found in the aerial parts is higher on a mg/g DW basis than that previously reported[2,4,9,32,34] while our results show similarity to the amounts reported (4–25 mg/g DW) by Kiferle[6] for leaves obtained from hydroponically grown plants.

The utility of the aerial parts of *Ocimum basilicum* and other plants belonging to the Lamiaceae family are well recognized for their RA content,[48] but to our knowledge only three studies, namely, Kiferle et al.,[6] Scagel and Lee,[34] and Toussaint et al.,[49] have studied roots as a source of RA. Interestingly, in our study roots of B13 in both systems showed an equal content of RA.


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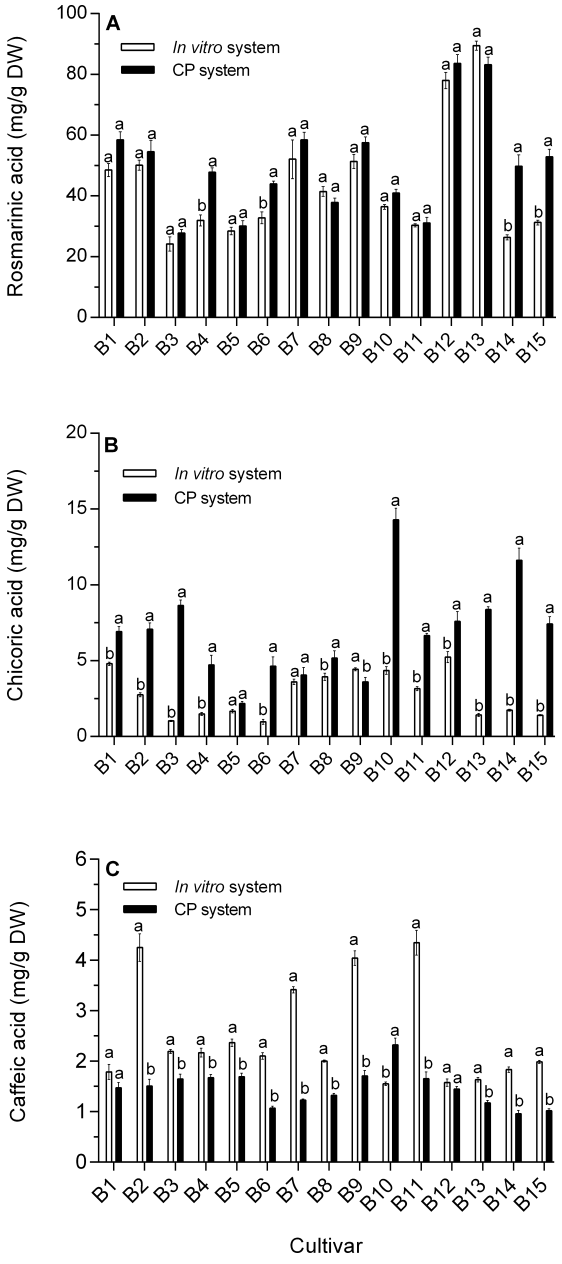


**Figure 5.** Concentrations of polyphenolics detected by HPLC analysis in the 15 *Ocimum basilicum* cultivars for the whole plant grown in the *in vitro* system and CP system. Comparison of (A) rosmarinic acid, (B) chicoric acid, and (C) caffeic acid (mg/g DW) content between the two growth systems. Data are presented as mean ± SEM of each of three replicates ($n$ = 3) for the whole plant. Different letters indicate significant differences ($p \leq 0.05$) between the two growth systems, within cultivars according to Sidak's multiple comparison test.

On the other hand, roots of cultivars such as B6, B3, B7, and B5 grown in the in vitro system produced up to three times more RA than in the CP system. Roots are clearly an alternative and rich source of RA. Kiferle et al.[6] reported high values of RA in roots of three cultivars at full bloom for plants growing in a hydroponic system and showed levels of RA similar to those found for two cultivars used in our study (B14, 27.32 ± 0.94 mg/g DW, and B7, 21.32 ± 0.85 mg/g DW). B13 in the in vitro system had RA content in the aerial parts of 45.33 ± 0.65 and in the roots of 44.11 ± 0.87 mg/g DW, showing that it has great potential as a source of RA and for selection studies. For all cultivars, similarity in RA profile for 11 cultivars between the CP system and the in vitro system shows that the in vitro system is very useful as a screening and selection tool for RA.

While the focus of this research was on quantification of RA in cultivars of *Ocimum basilicum*, the analysis of ChA and CA also offered insights into the contribution of other polyphenolics in the same cultivar and their antioxidant potential. Unlike RA, ChA content (Figure 5B) showed significant ($p < 0.05$) differences between both of the growth systems for all cultivars except for B5, B7, and B9. In the CP system B3 showed 8 times higher levels of ChA than in the in vitro growth system. The amount of ChA for the whole plant found in the in vitro growth system ranged from the highest level (5.24 ± 0.37 mg/g DW ChA) in B12 to the lowest level (0.98 ± 0.16 mg/g DW ChA) in B7. B12 (5.24 ± 0.37 mg/g DW ChA), B1 (4.80 ± 0.11 mg/g DW ChA), and B9 (4.44 ± 0.10 mg/g DW ChA) were found as high ChA producing cultivars in the in vitro system while B6 (0.98 ± 0.16 mg/g DW ChA), B3 (1.04 ± 0.04 mg/g DW ChA), and B15 (1.41 ± 0.04 mg/g DW ChA) were found as low producing cultivars. Similar to RA, aerial parts were found to be major sources of ChA in both systems (Tables 3 and 4). The amount of ChA (7.59 ± 0.37 mg/g DW ChA) found in B12 for the CP system and the amount found, 5.24 ± 0.37 mg/g ChA DW, in the in vitro system showed that this cultivar can be selected as a common high yielding cultivar.

The quantities of ChA reported in the in vitro and CP systems are higher than that reported in five studies to date on *Ocimum basilicum* and ChA.[2−4,9,34] Concentrations of ChA found in our study for B10 for the CP system are as much as 3.9 and 4.5 times higher than that reported in two previous studies.[29,4] Difference in growing conditions, age of harvest, extraction procedure, and analytical conditions can be defined as possible reasons for such a variable observation.[50] Although plants were grown for the same duration as in the studies conducted by Lee and Scagel[2] and Scagel and Lee,[34] the use of fresh samples and differences in cultivar type and extraction procedures may account for the differences of their values (0.114−0.885 mg/g FW and 0.05−0.01 mg/g FW) in comparison to our study. To the best of our knowledge, we have reported for the first time ChA in roots of *Ocimum basilicum.* No ChA was found in roots of B4, while the highest levels were found in B1 (2.45 ± 0.12 mg/g ChA) for plants grown in the in vitro system.

For CA in the in vitro system (Figure 5C) higher concentrations were found than in the CP system except for B10, which produced 1.4 times more CA. Cultivar type showed a significant effect on CA in both of the growth systems. The values found in this study ranged from 4.35 ± 0.25 mg/g CA DW in B11 to 1.55 ± 0.04 mg/g CA DW in B10. In the in vitro system high levels of CA were found in B11 (4.35 ± 0.25 mg/g CA DW), B2 (4.25 ± 0.27 mg/g CA DW), and B9 (4.04 ± 0.15 mg/g CA DW) while the lowest values were reported in B10 (1.55 ± 0.04 mg/g CA DW), B12 (1.58 ± 0.07 mg/g CA DW), and B13 (1.63 ± 0.04 mg/g CA DW). A low level of CA in B12 and B13 cultivars corresponds to the highest levels of RA in the same cultivars. As CA is a precursor to RA, low levels of CA compared to RA may indicate high biosynthetic turnover of CA. Similarly to RA and ChA, the aerial parts showed higher production of CA in 13 of the cultivars. Another possibility that may account for the higher levels of RA and ChA found compared with the levels of CA is that polyphenolic synthesis is altered during maturation and this may also account for the low

Table 4. Average Individual Polyphenolic Content in the 15 Cultivars of *Ocimum basilicum* Grown in the *in Vitro* System[a]

| | rosmarinic acid (mg/g DW) | | chicoric acid (mg/g DW) | | caffeic acid (mg/g DW) | |
|---|---|---|---|---|---|---|
| code | aerial parts | roots | aerial parts | roots | aerial parts | roots |
| B1 | 26.92 ± 0.75 d | 21.64 ± 2.29 de | 2.35 ± 0.10 cd | 2.45 ± 0.12 a | 1.45 ± 0.14 def | 0.34 ± 0.02 f |
| B2 | 35.10 ± 1.57 bc | 15.02 ± 0.20 efg | 1.82 ± 0.07 de | 0.94 ± 0.07 c | 2.36 ± 0.18 bc | 1.89 ± 0.12 b |
| B3 | 6.36 ± 0.17 f | 17.83 ± 2.18 def | 0.71 ± 0.08 g | 0.33 ± 0.05 ef | 1.45 ± 0.02 def | 0.74 ± 0.04 cde |
| B4 | 29.62 ± 1.81 cd | 2.34 ± 0.36 h | 1.49 ± 0.10 ef | nil | 1.74 ± 0.06 de | 0.43 ± 0.03 ef |
| B5 | 10.10 ± 0.09 f | 18.36 ± 1.27 def | 1.29 ± 0.11 efg | 0.39 ± 0.03 de | 1.94 ± 0.06 cd | 0.42 ± 0.02 ef |
| B6 | 7.93 ± 0.92 f | 24.87 ± 1.24 cd | 0.63 ± 0.13 g | 0.35 ± 0.03 de | 1.67 ± 0.09 de | 0.43 ± 0.03 ef |
| B7 | 17.06 ± 1.91 e | 35.04 ± 4.47 b | 3.30 ± 0.19 ab | 0.31 ± 0.02 ef | 2.60 ± 0.07 b | 0.81 ± 0.04 cd |
| B8 | 25.79 ± 0.70 d | 15.70 ± 1.10 efg | 2.98 ± 0.16 abc | 0.96 ± 0.09 c | 1.51 ± 0.04 de | 0.49 ± 0.03 def |
| B9 | 38.87 ± 1.97 b | 12.52 ± 0.78 fg | 2.27 ± 0.01 cd | 2.17 ± 0.11 a | 1.46 ± 0.02 def | 2.58 ± 0.16 a |
| B10 | 26.97 ± 0.49 d | 9.47 ± 0.72 gh | 3.35 ± 0.23 ab | 1.01 ± 0.04 c | 1.00 ± 0.02 fgh | 0.55 ± 0.03 def |
| B11 | 16.64 ± 0.77 e | 13.70 ± 0.61 efg | 2.71 ± 0.16 bc | 0.45 ± 0.04 de | 3.56 ± 0.23 a | 0.79 ± 0.06 cd |
| B12 | 47.79 ± 2.33 a | 30.21 ± 0.43 bc | 3.47 ± 0.27 a | 1.78 ± 0.10 b | 0.59 ± 0.03 h | 0.98 ± 0.05 c |
| B13 | 45.33 ± 0.65 a | 44.11 ± 0.87 a | 0.95 ± 0.08 fg | 0.47 ± 0.06 de | 0.93 ± 0.01 gh | 0.71 ± 0.03 cde |
| B14 | 17.51 ± 0.22 e | 8.89 ± 0.80 gh | 1.05 ± 0.08 fg | 0.69 ± 0.04 cd | 1.47 ± 0.04 def | 0.36 ± 0.01 f |
| B15 | 17.17 ± 0.73 e | 14.10 ± 0.53 efg | 1.09 ± 0.07 fg | 0.33 ± 0.04 ef | 1.35 ± 0.07 efg | 0.64 ± 0.08 def |

[a]Data represented as mean ± SEM of each of three replicates ($n = 3$) at a concentration of mg/g DW. Different letters indicate significant differences ($p \leq 0.05$) according to Tukey's HSD.

Table 5. Average Individual Polyphenolic Content in the 15 Cultivars of *Ocimum basilicum* Grown in the CP System[a]

| | rosmarinic acid (mg/g DW) | | chicoric acid (mg/g DW) | | caffeic acid (mg/g DW) | |
|---|---|---|---|---|---|---|
| code | aerial parts | roots | aerial parts | roots | aerial parts | roots |
| B1 | 39.60 ± 2.61 ab | 18.85 ± 0.25 c | 5.60 ± 0.24 cdef | 1.33 ± 0.08 cd | 1.18 ± 0.11 bc | 0.30 ± 0.01 fg |
| B2 | 32.69 ± 2.46 bcd | 21.83 ± 1.51 bc | 6.20 ± 0.26 cd | 0.88 ± 0.05 def | 1.00 ± 0.09 bcde | 0.50 ± 0.06 abcde |
| B3 | 16.11 ± 0.53 ghi | 11.71 ± 0.81 de | 6.89 ± 0.09 bc | 1.75 ± 0.13 c | 0.95 ± 0.10 bcde | 0.70 ± 0.03 a |
| B4 | 28.26 ± 1.31 def | 19.52 ± 0.70 c | 4.09 ± 0.37 fgh | 0.63 ± 0.01 ef | 1.15 ± 0.03 bcd | 0.52 ± 0.03 abcde |
| B5 | 13.60 ± 1.45 hi | 16.53 ± 0.58 cd | 1.65 ± 0.06 i | 0.53 ± 0.01 f | 1.19 ± 0.09 b | 0.50 ± 0.02 abcde |
| B6 | 32.04 ± 0.95 bcd | 11.92 ± 0.38 de | 3.66 ± 0.38 gh | 0.97 ± 0.04 def | 0.69 ± 0.01 efg | 0.38 ± 0.04 efg |
| B7 | 37.17 ± 1.69 abc | 21.32 ± 0.85 c | 3.47 ± 0.31 gh | 0.59 ± 0.07 ef | 0.80 ± 0.02 cdef | 0.42 ± 0.02 cdefg |
| B8 | 20.40 ± 0.96 fgh | 17.46 ± 1.10 cd | 4.45 ± 0.20 efg | 0.73 ± 0.09 def | 0.86 ± 0.03 cdef | 0.46 ± 0.01 bcdef |
| B9 | 36.29 ± 0.88 abcd | 21.23 ± 1.52 c | 2.80 ± 0.10 hi | 0.81 ± 0.07 def | 1.10 ± 0.08 bcd | 0.61 ± 0.05 abc |
| B10 | 30.52 ± 0.96 cde | 10.46 ± 0.39 e | 12.59 ± 0.30 a | 1.70 ± 0.14 c | 1.93 ± 0.14 a | 0.40 ± 0.02 defg |
| B11 | 11.60 ± 0.80 i | 19.52 ± 1.21 c | 5.47 ± 0.22 cdef | 1.18 ± 0.13 cde | 1.22 ± 0.10 b | 0.43 ± 0.03 cdefg |
| B12 | 44.71 ± 1.62 a | 38.89 ± 1.85 a | 3.43 ± 0.18 gh | 4.16 ± 0.23 a | 0.85 ± 0.02 bcdef | 0.60 ± 0.05 abc |
| B13 | 39.40 ± 1.74 ab | 43.84 ± 2.13 a | 4.66 ± 0.18 defg | 3.72 ± 0.07 ab | 0.53 ± 0.01 gh | 0.65 ± 0.04 ab |
| B14 | 22.46 ± 2.97 efg | 27.32 ± 0.94 b | 8.38 ± 0.75 b | 3.25 ± 0.29 b | 0.36 ± 0.05 h | 0.59 ± 0.08 abcd |
| B15 | 30.78 ± 1.46 cde | 22.08 ± 1.10 bc | 5.71 ± 0.27 cde | 1.71 ± 0.07 c | 0.77 ± 0.03 def | 0.25 ± 0.01 g |

[a]Data represented as mean ± SEM of each of three replicates ($n = 3$) at a concentration of mg/g DW. Different letters indicate significant differences ($p \leq 0.05$) according to Tukey's HS.

concentration of caffeic acid in comparison to RA and ChA as observed in our study.[18]

In the CP system plants were grown in the natural conditions and the light exposure was given equally and consistently to every unit by regular shifting. Similarly, in the in vitro system plants were grown under cool white fluorescent lights having qualities close to natural light and every unit was provided with uniform and consistent light conditions by regular shifting. Since the light conditions were consistent and the medium was consistent in each system, the polyphenolic response produced in the present study can be considered due to plant property in relation to the medium and light conditions. Additionally, the objective of the present work was to compare and find out whether an alternate system to the CP system can be evolved, and as the results have shown a similar trend for the major polyphenolic (RA), we presume that the developed technique is true to type to a greater extent.

Significant differences were found between the two growth systems for the four cultivars in RA and 13 out of 15 cultivars in ChA and CA profile. Conventional pot system was found to have higher amount of RA and ChA. Differences in the growth substrates, regular nutrient supplementation, and early flowering for all cultivars growing in conventional pot system can be hypothesized as the reasons for high RA and ChA content in the CP system and its significant difference from the in vitro system.[9,29,32] Additionally, as the extraction methodology was optimized for RA in the present study, it can be concluded that separate and targeted extraction of ChA and CA is needed to correlate the effects of growth substrate, growth parameters, and extraction methodology on their content in the two systems.

In the present study chicoric and caffeic acids are the minor polyphenolics found in the cultivars of *Ocimum basilicum*. The CP system is found as a better system for ChA while the in vitro system is found to be better for CA, clearly indicating that the amount of minor polyphenolic is differently affected by the growth substrate and growth parameters in basil in comparison to the major polyphenolics such as RA. The reasons for such an

observation can be studied in the future with extraction methodology optimized for these minor metabolites. It can be further postulated that parameters such as light quality, humidity, and temperature strongly affect the biosynthetic pathway for expression of minor polyphenolics in basil.

*Total Antioxidant Potential and Individual Polyphenolic Antioxidant Potential.* Antioxidant potential can be defined as the capacity of chemical moieties to act as inhibitors of free radicals, donators of hydrogen, quenchers of singlet oxygen, or molecules which can interrupt the oxidation process.[7] Thus, the identification of antioxidants from complex matrices is of significant importance to food, agriculture, and pharmaceutical industries. Detection of antioxidants by conventional antioxidant assays such as DPPH and FRAP assay is time-consuming. Acidic potassium permanganate reacts with potential antioxidant molecules to produce light due to excitation of manganese II species. The signals produced by the acidic potassium permanganate assay show good agreement with conventionally used assays thus its use has been advocated as an excellent alternative.[35]

Polyphenolics react with acidic potassium permanganate,[41] which is a distinct advantage of the chemiluminescence based assay over conventional assays. The method used in our study for total antioxidant assessment was as described by McDermott et al.[36] but modified and applied, for the first time to our knowledge, to *Ocimum basilicum* plant extracts. Total antioxidant content varied among the 15 cultivars for both growth systems. For the in vitro grown plant samples antioxidant potential was higher in comparison to CP grown plants. Similarly, in terms of total antioxidant content (Figure 6) no significant difference was found for the same 11 cultivars

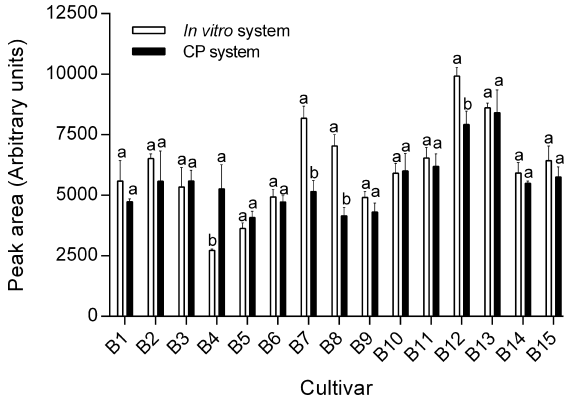


**Figure 6.** Total antioxidant potential found in 15 *Ocimum basilicum* cultivars grown in the *in vitro* system and the CP system by chemiluminescence analysis. Total antioxidant potential is expressed in terms of peak area. Data are presented as mean ± SEM of each of three replicates ($n$ = 3) for the whole plant. Different letters indicate significant differences ($p \leq 0.05$) between the two growth systems, within cultivars according to Sidak's multiple comparison test.

grown in both of the growth systems. B13 and B12 cultivars showed the highest chemiluminescence signals for plants growing in both growth systems.

It is important to identify individual polyphenolics within a complex matrix which may be responsible for antioxidant activity. To assess the contribution of individual polyphenolics, an enhanced acidic permanganate assay, a postcolumn technique, coupled with HPLC was performed on extracts from all cultivars growing in the in vitro growth system. RA antioxidant detection by the acidic potassium permanganate assay has been reported earlier by our group,[35,36] and in the present study its contribution to total antioxidant content was determined by comparing the peak area of RA with the peak area of total antioxidants. The highest antioxidant activity was found in association with RA for high yielding cutlivars such as B13 (Red Rubin), B12 (Holy Green), and B7 (Basil Genovese) cultivars growing in the in vitro system. RA associated antioxidant activity contributed to 44.7%, 44.4%, and 33.6% of the total antioxidant potential in these three cultivars while ChA (2.8%, 3.9%, and 5.8%) and CA (1.0%, 1.1%, and 1.7%) showed a lower percentage of total signal. RA is the major antioxidant compound detected in this study followed by ChA and CA. A positive correlation between the total polyphenolic content (RA + ChA + CA) detected by HPLC and the total polyphenolic antioxidant potential (RA + ChA + CA) and antioxidant potential of RA detected by chemiluminescence was found in this study (Figure 7). The presence of two catechol

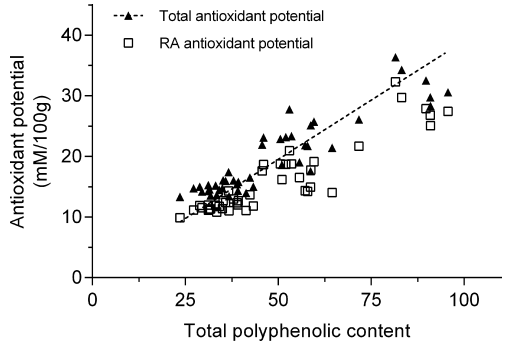


**Figure 7.** Correlation between antioxidant potential (total polyphenolic antioxidant potential and rosmarinic acid antioxidant potential) detected by chemiluminescence assay and total polyphenolic content detected by HPLC analysis for the 15 *Ocimum basilicum* cultivars grown in the in vitro system. The Pearson correlation coefficient ($r$) for antioxidant potential with total polyphenolic content response was 0.910 and 0.912 ($R^2$ = 0.828, 0.832) respectively. Antioxidant potential detected by chemiluminescence assay is expressed as mM/100 g. Data are presented as mean ± SEM of each of three replicates ($n$ = 3) for the whole plant.

rings with a carboxylic acid group gives rise to the antioxidant potential of RA.[5] The acidic potassium permanganate chemiluminescence detection and the subsequent determination of the antioxidant characteristics of ChA were observed for the first time in this study. The chemiluminescence assay is a sensitive and efficient method for determination of antioxidant activity that we propose as a tool for quick selection and screening of conventionally or in vitro grown samples. Our results indicated that polyphenolics content and antioxidant potential detected by chemiluminescence were positively correlated and significantly affected by cultivar type.

**Correlation between Morphotype and Individual Polyphenolic Content.** The contents of RA, ChA, and CA in aerial parts and roots of all cultivars were subjected to hierarchical cluster analysis to examine similarities and differences between the cultivars. Two distinct groups were generated based on the polyphenolic content analysis (Figure 8), and within the two main groups several subgroups were evident. Two main groups were primarily separated on flower color, which was highly correlated with plant morphology (leaf shape, leaf surface, stem type, plant habit) and RA content.

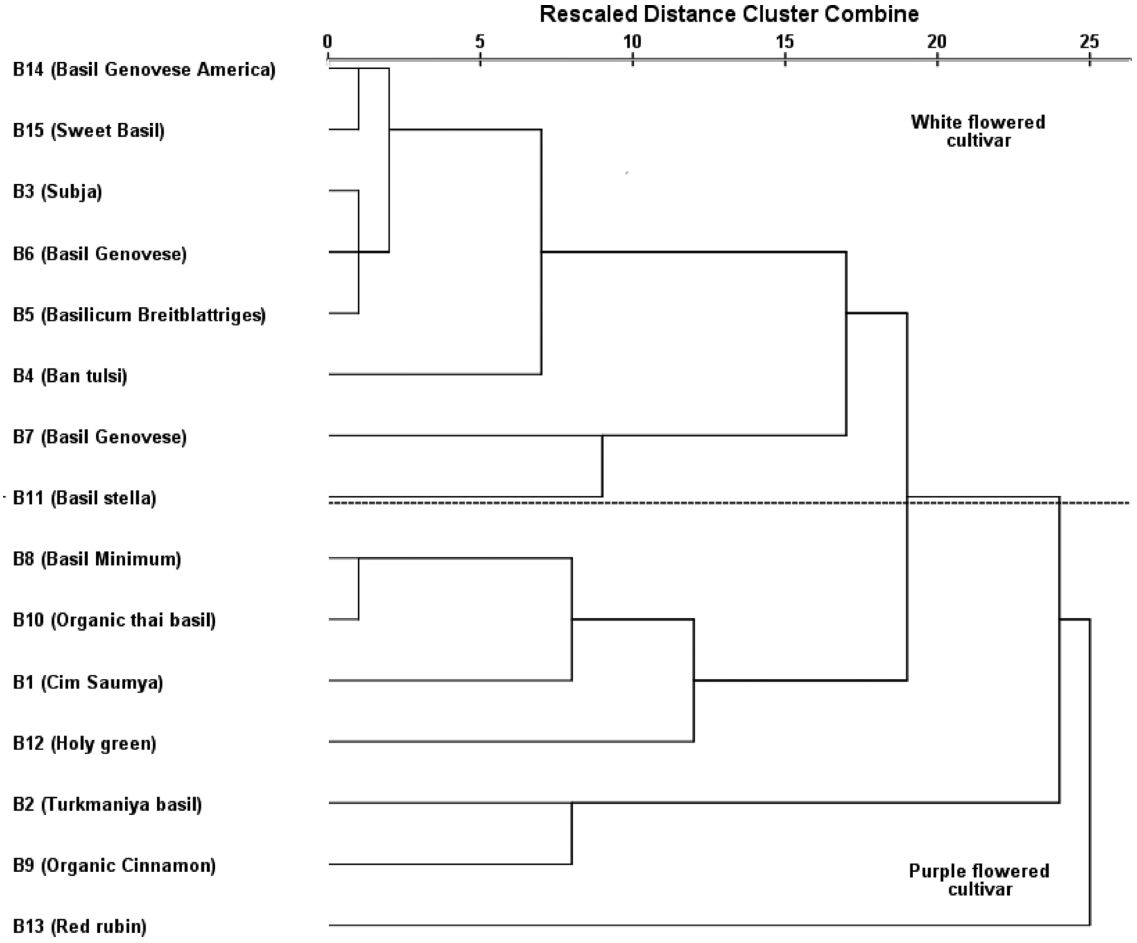


**Figure 8.** Clustering of 15 *Ocimum basilicum* cultivars grown in the *in vitro* system based on rosmarinic acid, chicoric acid, and caffeic acid content found in aerial parts and roots. Data used was standardized with *Z*-score using squared Euclidean distance, and dendrogram was drawn using SPSS 22 statistical computer package. Purple flowered cultivars showed higher contents of rosmarinic acid in comparison to white flowered cultivars. Dashed lines represent the division between the white and purple flowered cultivars.

Those cultivars with the highest RA content were purple flowered, and those with lower RA content were white flowered except for B4, which is a purple flowered cultivar that fell into the white flowered cluster.

**Merits of the in Vitro System.** The advantages of the developed in vitro screening system over conventional pot system include use of sterile, transparent (ease of observation), and precisely defined medium for root growth, no additional nutrient supplementation, and less labor and physical space requirement. These advantages allow for ease of downstream processing and nondestructive sampling for fast and reproducible screening of officinal plants. Additionally, the proposed in vitro system also shows advantages over hydroponics as a screening system as it does not requires use of specialized instrumentation, technical skills, labor, and energy.

Our study identified B13 (Red Rubin), B12 (Holy Green), and B7 (Basil Genovese) as high yielding cultivars in terms of high polyphenolic (RA) content and total polyphenolic antioxidant potential. Rosmarinic acid is the major metabolite found in the cultivars tested, and we have discovered that, in addition to its presence in the aerial parts of plants, roots are a viable and alternative source of RA. A distinct relationship in morphology and polyphenolic content was also revealed in this study, and purple flowered cultivars were found to be a rich source of RA compared with white flowered cultivars. We propose that the screening method described here will be applicable for the screening of a wide variety of secondary metabolites in a range of herbaceous annuals and that the method lends itself to the rapid determination of antioxidant activity in plant extracts.

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## ABBREVIATIONS USED

CP, conventional pot; RA, rosmarinic acid; ChA, chicoric acid; CA, caffeic acid; OPA, *o*-phosphoric caid; DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; ABTS$^{\bullet+}$, 2,2′- azinobis(3-ethylbenzothioazoline-6-sulfonic acid) radical cation; HPLC, high performance liquid chromatography; GAE, gallic acid equivalents

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