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ESTIMATION OF SALICYLIC ACID IN EUCALYTPUS LEAVES USING SPECTROPHOTOMETRIC METHODS

Warrier R . R.**, *M. Paul, M. V. Vineetha

Institute of Forest Genetics and Tree Breeding, Forest Campus, PB 1061, R. S. Puram Coimbatore-641002, Tamilnadu, India

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Summary: A simple and reliable procedure for spectrophotometric determination of salicylic acid (SA) in Eucalyptus leaves is described. The procedure is based on the formation of $Fe(H_2O)_6^{3+}$ ion which has an intense violet colour when SA in an aqueous form reacts with Fe (III). The absorbance of the complex was measured at 540 nm. The procedure was tested with different solvents and varying sample sizes to optimize the extraction protocol and study the interference of oils.

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INTRODUCTION

Plants have been subjected to attack by an array of organisms like viruses, bacteria, fungi, insects, nematodes and herbivores for centuries. In their long association with pests and pathogens, plants have evolved an impressive array of defensive tools. The evolution of chemical defenses in plants is linked to the emergence of chemical substances that are not involved in the essential photosynthetic and metabolic activities. These substances, secondary metabolites, are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms, and are often produced as by-products during the synthesis of primary metabolic products. These secondary metabolites play a major role in defense mechanisms against herbivores.

Salicylic acid (SA) or ortho-hydroxy benzoic acid and related compounds belong to a diverse group of plant phenolics. Salicylates from plant sources have been used in medicine since antiquity. Using modern analytical techniques it has been found that salicylates are distributed in many important agricultural plant species. SA is a phenolic phytohormone playing a role in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA also induces specific changes in leaf anatomy and chloroplast structure.

*Corresponding author: rekhawarrier@gmail.com, rekha@icfre.org

The discovery of flower-inducing action and bud formation in tobacco cell cultures by Eberhard et al. (1989) was the first indication of a physiological effect of SA. The stimulatory effect of SA on flowering was later demonstrated in other plant species forming the basis for suggesting that SA can function as an endogenous regulator of flowering (Cleland and Ajami, 1974).

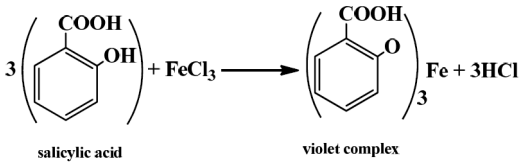
SA is involved in endogenous signaling, mediating plant defense against pathogens. It plays a role in the resistance to pathogens by inducing the production of pathogenesis-related proteins. It is involved in the systemic acquired resistance (SAR) in which a pathogenic attack on one part of the plant induces resistance in other parts. The signal can also move to nearby plants by SA being converted to a volatile ester, methyl salicylate. The highest levels of SA were determined in the inflorescence with necrotizing pathogens (Raskin, 1992).

SA is a relatively polar, poorly aqueous soluble material. The salt form, however, is water soluble. Further, it can be easily extracted into water at high temperatures. Its presence can be detected in very minute quantities of 1 part to 100,000. A colorimetric method by ferric chloride, comparing the depth of color from a known solution of SA, was given by Muter (1876) for food samples. SA, with ferric chloride, has also been proposed by Weiske (Cohn, 1899) as an indicator in acidimetry. Routine analysis of the compound has been carried out in food, dairy and pharmaceutical industry (AOAC, 1967; Venema et al., 1996; FSSAI, 2012). However, in plants sophisticated methods like the thin-layer densitometric method (Chrastil and Wilson, 1978), gas chromatography-mass spectrometry (Meuwly et al., 1995); HPLC-based method involving extraction of SA in organic solvents, evaporation of organic solvents, chromatographic purification and detection by fluorescence spectroscopy (Verberne et al., 2002; Abdoul-Sood et al., 2004); biosensor-based method and HPLC-based approach (Marek et al., 2010); gas chromatograph with electron capture detection method (Meher et al., 2011) are available, but they are extremely costly and time consuming.

Eucalyptus clones are commercially planted because of their valuable wood and fiber properties which have been exploited in the pulp and paper industry. Although Eucalyptus trees are generally disease tolerant, they can do succumb to diseases caused by a wide range of pathogens (Wingfield et al., 2008). Jacob et al., (2007) reported a severe outbreak of an invasive insect, *Leptocybe invasa*, presumed to have made an entry from Australia, on eucalypts plantations and nurseries all over the country. This tiny wasp has been reported to induce galls on shoot terminals, on petioles and midribs in saplings and trees of eucalypts (Mendel et al., 2004). A report on the mode of action of the wasp by Krishnakumar and Jacob (2010) suggested biotrophic pathogenesis which triggered the SA pathway for defense mechanisms. A stepping stone for improving our understanding of Eucalyptus responses to pathogen defenses would be to quantify the levels of SA in these plants. Since no standard protocols are available for quantitative estimation of SA in tree species, the aim of this study was to standardize an extraction and estimation protocol for free SA in the leaves of Eucalyptus trees.

MATERIALS AND METHODS

SA easily forms complexes with minute traces of ferric salts and hence, can be used as a very useful method for detection of the compound. It forms a violet complex due to the formation of a ligand and the extinction of the ferric complex can be determined at 540 nm. This principle was adopted in the present study for the detection of SA in Eucalyptus.



Standard solutions

SA reacts with almost all solvents and has high solubility. It is soluble in many solvents. One gram can be dissolved in 460 ml of water at room temperature, 15 ml boiling water, 2.7 ml alcohol, 3 ml acetone, 42 ml chloroform, 135 ml benzene, 60 ml glycerol, etc. (Rainsford, 2004). Standards of varying concentrations were prepared in chloroform, amyl alcohol, ether, ethanol and water to check the suitability of the solvents for extraction of SA.

A. Water as solvent - Stock SA solution: 100 mg SA was dissolved in sufficient water to make 100 ml of solution. This solution contained 1000 ppm. Working Stock solution: 1.0 ml of the stock solution was added to sufficient water to make 10 ml of the solution. This solution contained 100 ppm (µg/ml).
B. Organic Solvents (chloroform, amyl alcohol, ether, ethanol) - Stock SA solution: 100 mg of SA was dissolved in 100 ml of solvent. This solution contained 1000 ppm. Working Stock solution: 1.0 ml of the stock solution was added to the solvent to make 100 ml of the solution. This solution contained 1000 ppm (µg/ml).

All stock and standard solutions may stay for at least 6 months if stored in a refrigerator.

Optimization of extraction protocols

Leaf samples of Eucalyptus were frozen in liquid nitrogen and ground to powder. Samples were left at room temperature to thaw. Varying aliquots (10 – 1000 mg) of the sample were extracted in 1.0 ml of different solvents to assay the solubility of SA from tissues in the presence of interfering substances. Eight clones of Eucalyptus were chosen to study the pattern of variation in levels of SA following the optimized sample weight and extraction volume. This was done to optimize the solvent to be taken up for extraction. This was followed by extraction of SA into the optimized solvent by varying the volumes of the solvents for 50 mg and 100 mg tissue.

In all experiments, samples were swirled well in the solvent followed by centrifugation at 10,000 g for 10 min. The supernatant was stored on ice for SA measurement. 100 µl of the supernatant was mixed with 0.1% freshly prepared ferric chloride. The volume of the reaction mixture was made up to 3.0 ml and the complex formed between Fe^{3+} ion and SA, which is violet in colour was determined by spectrophotometry, measuring the absorbance of the complex in the visible region (at 540 nm).

SA measurements were carried out with different clones of Eucalyptus using the standardized protocols and the amount of SA in the leaf samples was determined accordingly.

Statistical analysis

A single-factor analysis of variance (one-way ANOVA) was adopted to investigate the effect of different solvents on the extraction of SA and compare any significant differences between them. Experimental results were means of five parallel measurements and were analyzed by Microsoft Excel 2010 and SPSS 16.00 for Windows. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Development of standard curves

Five different solvents which have been reported as solvents for SA were used for the generation of the standard curves. It was observed that it took longer time to dissolve SA in water which could be easily dissolved by slight warming of the solution. SA was dissolved rapidly in organic solvents. For the development of the standard curves, it was observed that water as a solvent was aliquoted in 100, 200,…500 µg series while in case of organic solvents, detection was observed in 20, 40, …100 µg series. Standard curves of SA using the five different solvents and the curve of best fit by regression analysis were worked out. The calibration curve, curve of best fit and regression coefficients are presented in Fig. 1 A–E. All standard graphs had an r^2 value closer to 1 indicating strong correlation between the x and y axis data.

Development of extraction protocols

Once the calibration equations for the five solvents were developed, they were used to estimate the concentration of SA in Eucalyptus samples. Samples ranging from 10 mg to 1.00 g were ground in 1.0 ml of different solvents and the absorbance was measured at 540 nm (Fig. 2). It was observed that although the standard curves obtained for SA dissolved in organic solvents showed intense colour at low concentrations, these solvents were not able to effectively extract SA from the plant leaf samples. Among the different organic solvents, ethanol showed slightly improved results. However, it was observed that after extraction of free SA in water (aqueous extract), maximum SA in the samples was detected. There was a steady and linear increase in the levels of SA in the aqueous extract from 10 mg to 50 mg which plateaued at 60 and 70 mg. This was followed by a gradual reduction in the levels of SA suggesting that with increasing sample size, the efficiency of SA extraction into the solvent was reduced. A similar trend was also observed in ethanol. With increasing concentrations in the sample, the solvent got saturated with SA resulting in a decline in the levels of SA with increasing sample size.

Eight clones of Eucalyptus were selected for extraction of SA using different solvents with the standardized sample weight and volume. ANOVA analysis revealed that significant differences were observed in the levels of SA extracted from the different clones using different solvents (Table 1). Maximum extraction was observed in an aqueous solution followed by ethanol. Extractions in acetone, amyl alcohol and chloroform were on par. This suggests interference of fats/oils in the extraction of SA in the organic solvents tested.

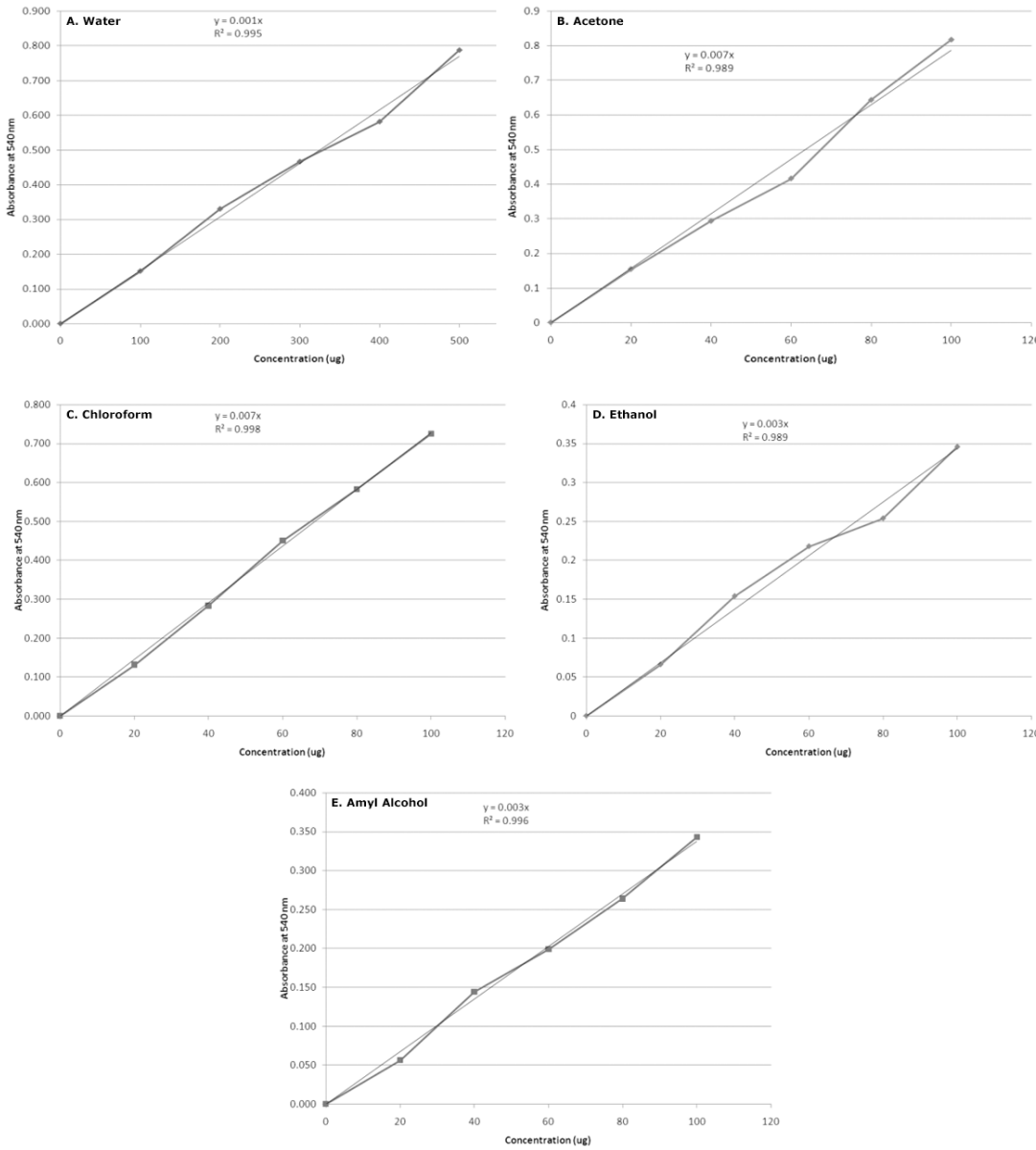


Figure 1. Standard curves of SA using different solvents.

Optimization of the extraction methods

To fine tune the quantity of samples required for effective extraction of SA from the leaves of Eucalyptus, two samples (50 and 100 mg) were taken and extracted in varying volumes of water ranging from 100 µl to 10.0 ml (Fig. 3). It was observed that in the 50 mg sample, there was a steady increase in the amount of SA from 100 to 1000 µl which declined with further increasing the volume of water whereas in the case of 100 mg sample, SA was found to be constant till the volume reached 1000 µl followed by a drastic reduction. T-test at 5% level for comparing the two samples revealed that the variations were insignificant suggesting that 50–100 mg

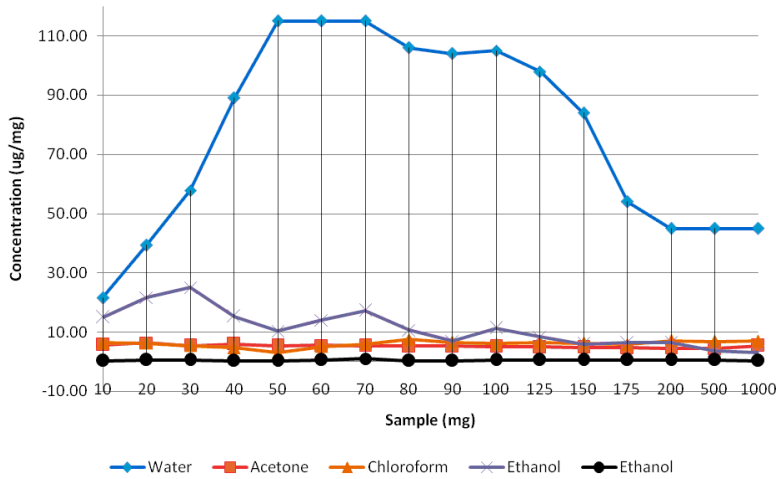


Figure 2. Development of protocols for extraction of SA from Eucalyptus leaves using five different solvents.

Table 1. ANOVA for SA estimation in different clones of Eucalyptus using different solvents.

	Source of Variation	SS	df	MS	F	P-value
Clones	Between Groups	64727.3	7	9246.76	0.151	0.993
	Within Groups	1965842	32	61432.6		
	Total	2030569	39			
Solvents	Between Groups	1727085	4	431771	49.795	0.000
	Within Groups	303484	35	8670.97		
	Total	2030569	39			

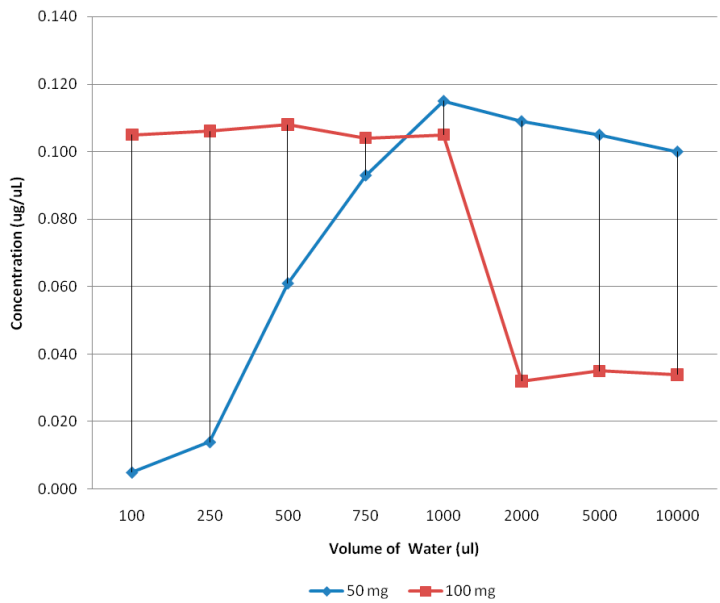


Figure 3. Optimization of sample size and extraction volume for optimal extraction of SA from Eucalyptus leaves.

sample could be the optimal sample size for extraction of SA from Eucalyptus leaves.

CONCLUSIONS

1. In this study, a rapid method for determination of SA in Eucalyptus leaves based on its reaction with ferric chloride, which reacts with minute traces of SA to yield a purple coloured complex was presented.
2. The effect of some possible interfering substances, mainly oils, which are present abundantly in Eucalyptus, rendered extraction of SA in organic solvents ineffective.
3. For extraction, 50-100 mg sample in 1.0 ml of water provided repeatable results.
4. In comparison with the well known sophisticated methods like HPLC, GC, MS where extraction procedures are very cumbersome and time-consuming, the described method is simple, fast, reliable and accurate.

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