COMPARATIVE EVALUATION OF LIMONIA ACIDISSIMA L. GROWING IN VIVO AND IN VITRO FOR ASCORBIC ACID CONTENT

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ABSTRACT

In the present investigation *Limonia acidissima* was screened for ascorbic acid content from various plant parts and tissue cultures. Different plant parts (Leaves, bark , fruit shell, pulp and seeds) of *Limonia acidissima* were procured from wild regions of Bikaner and Bharatpur districts (Rajasthan). *In vitro* cultures were established on MS medium supplemented with various concentrations and combinations of PGRs. Maximum amount of ascorbic acid was recorded in pulp (63.80 ± 0.054mg/100 gwd and callus (63.76± 0.012 mg/100gwd) in sample BIK-1. Minimum amount of ascorbic acid (44.15± 0.043 mg/100gwd) was found in bark of sample BRP -2. Comparable amount of ascorbic acid concentration was found in all the samples tested.

KEY WORDS: Ascorbic acid, *Limonia acidissima* L., Plant growth regulators, *In vitro* culture.

1. Introduction

Limonia acidissima L. is an underexploited potential tree whose fruits are edible and other parts also have potent traditional applications. *Limonia acidissima* L. as a whole, or its parts such as unriped fruit, riped fruit, root, bark, trunk gum and leaves have a broad spectrum of traditionally established therapeutic properties [1] and widely used in several Ayurvedic preparations like Panch kapittha and Kapitthaashtaka churna. The fruit is recommended in Ayurveda for treatment of tumors, asthma, wounds, diarrhea, dysentery, cardiac dysfunction, hepatitis, sore throat, ophthalmia and leucorrhoea etc. Fruit pulp is used to treat certain ailments like diabetes [2] and gastric ulcers. Seeds are used in heart diseases. The leaves are astringent and carminative; good for vomiting, indigestions, hiccup, ulcer and dysentery [3]. Leaf extract of the plant has antioxidants[4], larvicidal [5], antidiabetic[2] and hepatoprotective[4] potentials. It is very rich source of vitamins and bioactive compound, especially vitamin C, B, thiamine, riboflavin and alkaloids but it has not been much studied. Ascorbic acid or vitamin C is an important primary plant product and is claimed as a 'cure all ' for many human diseases. Increased intake of vitamin C, an important antioxidant found in fruit and vegetables, is strongly linked to reduced risk of many types of cancers [6]. It is also known as anti-scorbutic metabolite because of its curing action against scurvy. In humans it has several critical functions as an enzyme Co-factor. It participates in collagen synthesis, carnitine synthesis, converting dopamine to noradrenalin and Cholesterol metabolism besides its involvement in the immune system [7]. It is a potent electron donor and reducing agent and also acts as water soluble antioxidant. The determination of ascorbic acid has gained increased significance in several areas of analytical chemistry such as pharmaceutical, clinical and food application. Keeping in view its importance, present study was undertaken with objective to evaluate the ascorbic acid content in *Limonia acidissima* growing at two different ecological sites in Rajasthan and *in vitro.*

MATERIALS AND METHODS

2.1 Collection of plant material

Leaves, bark, fruit shell, pulp and seeds of *Limonia acidissima* were procured from wild region of Bikaner and Bharatpur districts (Rajasthan) and the plant was botanically authenticated from Herbarium (Voucher specimen D.C.M.1850: DCB NO.-1219; BSI-3355), Department of Botany, Govt. Dunger college, Bikaner, Rajasthan.

2.2. Establishment of *in vitro* culture

Various explants *viz*. Epicotyls, hypocotyls, cotyledon and internodal segments were excised from 6-8 week old *in vitro* grown seedlings. These explants were then established and maintained by frequent subculturing after 4 weeks on MS Medium supplemented with various concentrations and combinations of kinetin and 2, 4-D for callus induction and kinetin and BAP for induction of multiple shoots. Cultures were maintained in growth chamber with regulated temperature (26±2^{o}C), relative humidity (55±5%), 3000 lux light intensity. Data was recorded after 2, 4, 6, 8 and 10 weeks and growth indices were calculated.

2.3. Establishment of Ascorbic acid

Different plant parts and cultures harvested at maximum GI were dried, powdered and screened for ascorbic acid content by following the procedure of Chinoy [8]. The cultures as well as different plant parts were weighed, crushed in ice cold CO_2 saturated water and the extract was made to a definite volume. 3 ml of extract was mixed with an equal volume of buffered metaphosphoric acid at pH 3.6. Two ml aliquot of this solution was mixed with 5 ml distilled water and the turbidity produced was adjusted to zero. Another 2 ml aliquot was then mixed with 5 ml of 2, 6-dichlorophenol indophenol, prepared by dissolving 5 mg in 100 ml of distilled water at 80^{0}C and the optical density was measured. Absorbance of each of the sample was measured on a VIS Spectrophotometer (Labtronics LT-391) set at 546 nm against blank. The amount of ascorbic acid present in 1 ml of the original extract was obtained by using the regression formula:

$$Y = 0.1103 – (0.14 \times O.D.)$$

Where,Y = Concentration of ascorbic acid in mg.

O.D. = Optical Density

From the contents of 1 ml of the extract the ascorbic acid content per 100 gm fresh weight was calculated as follows:

$$\text{Free ascorbic acid} = \frac{(A \times V)}{W} \times 1000 \times 100$$

Where, A = Y = mg ascorbic acid / ml of original extract

V = Total volume of the original extract (in ml)

W = Weight of the plant tissue sample (in mg), used for analysis

2.4. Statistical Analysis

All experiments are performed in triplicates. Data are represented as mean ± standard deviation.

3. Results and Discussion

Table1: Evaluation of ascorbic acid content (mg/100gwd) in *Limonia acidissima in vivo* and *in vitro*.

	Ascorbic acid content(mg/100gwd)						
Plant parts	Leaves	Fruit shell	**Pulp**	Seed	**Bark**	Callus	*In vitro* shoots
BIKS1	58.35 ± 0.008	60.46 ± 0.041	**63.80±0.054**	61.56 ± 0.016	**55.08 ± 0.129**	63.76±0.012	59.45 ± 0.008
BRPS2	57.29 ± 0.040	58.27 ± 0.037	**62.72±0.037**	56.04 ± 0.027	**44.15 ± 0.043**	62.65±0.029	58.37 ± 0.069

Values are expressed by Mean±SD of three replicates

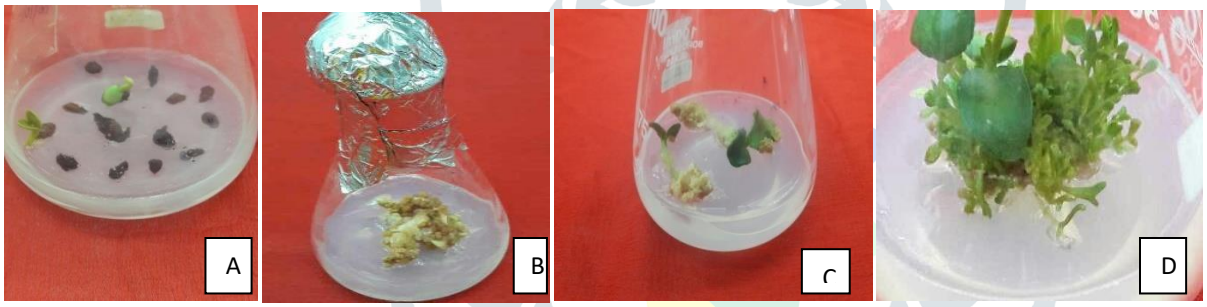

Fig 1- *In vitro* propagation of *Limonia acidissima* : A. *In vitro* grown seedling; B. callus on MS medium supplemented with Kinetin (0.5mg/L) and 2,4-D (1.0mg/L); C. Initiation of Shoot from hypocotyls on MS medium supplemented with Kinetin (0.5mg/L) and BAP (0.5mg/L); D. Multiple shoots on MS medium supplemented with Kinetin (0.5mg/L) and BAP (0.5mg/L).

Profuse callusing was observed in hypocotyls and epicotyls cultured on MS Medium Supplemented with 2,4-D (1.0 mg/L) and Kinetin (0.5mg/L). The callus was fragile, creamish green, fast growing and embryogenic. Best *in vitro* multiple shoot regeneration was observed in internodal segments cultured on MS medium supplemented with BAP (0.5mg/L) and Kinetin (0.5mg/L) as shown in figure -1.

Appreciable amount of ascorbic acid content was found in different plant parts as well as different samples of *Limonia acidissima* collected from Bikaner and Bharatpur districts as depicted in table - 1. Concentration of free ascorbic acid was found to be comparable in all the samples tested. Maximum and similar amount of ascorbic acid was recorded in pulp (63.80 ± 0.054mg/100gwd) and callus cultures (63.76± 0.012mg/100gwd) of BIK -1 sample (Bikaner district). The minimum amount of ascorbic acid was found in bark (44.15 ±

0.043mg/100gwd) of BRP-2 sample (Bharatpur district). Among all the plant samples compared maximum amount was observed in pulp and minimum amount was observed in bark. Except bark not much variation in ascorbic acid content was found in samples collected from two different ecological sites. *In vitro* tissue cultures (both callus cultures and *in vitro* shoots) also showed significant amount of ascorbic acid when compared with *in vivo* plant parts.

Kumar and Deen [9] reported 6.82 mg/100g ascorbic acid in fully ripen fruit of *Limonia acidissima.* Shrestha *et al.* [10] recorded ascorbic acid in *Citrus limon* (34.8 mg*), Citrus aurantium* (29.89 mg), *Citrus aurantium var. sinensis* (25.11 mg), *Citrus maxima* (61.29 mg), *Citrus paradise* (39.80 mg) and *Citrus medica* (17.4mg). Deekshika *et al.* [11] evaluated the ascorbic acid content in fruit & vegetable and reported highest amount of ascorbic acid in *Mangifera indica* 54.78 mg/100g , *Citrus tangerina* 47.84 mg/100g and *Citrus limetta* 47.41mg/100g. Maximum amount of ascorbic acid in fruits can be related to its reservoir nature [16]. However maximum amount of ascorbic acid has been reported in flowers of *Moringa oleifera* then leaves and pods [12]. Kapoor and Bansal [13] reported maximum (57.21 mg/100 g.d.w.) amount of ascorbic acid in fruits of *Salvadora ssspersica* and minimum in stem of *Tecomella undulata* (41.00 mg/100 g.d.w.). Najwa and Azrina [14] analysed vitamin C content through HPLC and revealed highest amount in orange (43.61 mg) followed by lemon, grapefruit , lime , and musk lime. Free endogenous ascorbic acid production *in vivo* and in tissue culture has been reported by many workers [15, 16, 17] .

4. Conclusion

Our findings revealed that there is not much variations in ascorbic acid content in *in vivo* plant parts as well as in *in vitro* cultures of *Limonia acidissima* collected from two different ecological sites and the plant is a rich source of ascorbic acid as compared to other citrus plants. The present study also supports that *in vitro* tissue cultures also possess biosynthetic potentialities to produce free ascorbic acid in good amount and promote the plant tissue culture technology in the area of the production of this small and familiar molecule. Thus this underexploited and undervalued plant is recommended for the use as dietary source of vitamin C.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ETHICS STATEMENT

We give our consent to participate under the ‘Ethics, consent, and permissions’ heading as applicable.

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