Micropropagation of *Eclipta alba* (L.) Hassk. and chemical profiling of cultures using HPTLC

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Abstract
In the present study, nodes of Eclipta alba (L.) Hassk. were placed in Murashige and Skoog's (MS) medium fortified with different concentrations of plant growth regulators (PGRs). Amongst all the combinations tried, Murashige and Skoog's (MS) medium fortified with N^6-benzyladenine (BA, 10 µM) and indole-3-acetic acid (IAA, 1 µM) formed maximum 18.40±0.84 shoots in 100% cultures. In vitro shoots derived from optimized medium were assessed for their biosynthetic potential using HPTLC fingerprinting which revealed that these shoots are also able to synthesize similar metabolites as in vivo shoots.

Further in vitro shoots were rooted using different strengths of static and liquid MS media as well as in natural planting substrates of which ½MS liquid medium fortified with sucrose (1%) and indole-3-butyric acid (IBA, 8 µM) induced optimum 40.50±1.38 roots within four weeks. The rooted shoots through in vitro and ex vitro rooting were successfully hardened and acclimatized in garden soil under greenhouse conditions.

Keywords: Clonal propagation, hardening, HPTLC fingerprint, medicinal plant, nodal explant.

Introduction

Eclipta alba (L.) Hassk. (Asteraceae), commonly known as bhringraj, is an annual herb and conventionally used in preparation of hair oil as a dyeing agent for hair blackening and prevents premature hair-fall[23]. The plant contains important metabolites like eclalbatin, α-amyrin, ursolic acid, oleanolic acid, saponin, daucosterol, wedelolactone and demethylwedelolactone[7,28,30]. It is used in the treatment of liver diseases[2,5,22] and also has anti-inflammatory, bronchodilator[10], hepatoprotective and anti-hyperglycemic activities[1]. Whole plant is consumed for preparation of herbal medicines and hair oils which requires more than 10 tons of plant material every year[29]. However, to fulfill the demand, it is indiscriminately harvested from wild which increases threat on wild population and creates gap between demand and supply.

The gap can be fulfilled by *in vitro* regeneration of plantlets[24,25], but the biosynthetic potential of *in vitro* plants may alter and can be assessed using chromatography techniques[8,14]. Fingerprinting is an accurate method for authentication as well as for identification of botanical materials and high performance thin layer chromatography (HPTLC) is reported to be one of the methods used for the same[4]. HPTLC fingerprint has been utilized by several researchers to assess the chemical integrity of *in vitro* regenerated plants[17,26].

The main aim of the present study was to establish efficient micropropagation protocol along with development of chemical fingerprints.

Material and Methods

Plant material and surface sterilization: Healthy nodes of *E. alba* (L.) Hassk. were collected from garden of Faculty of Science, The M.S. University of Baroda. They were kept in running water for 1 h, washed with labolene (Fisher Scientific, India) and surface sterilized using bavistin solution (0.01%) and $HgCl_2$ (Merck, India) solution (0.1%) for 4 min each before inoculation.

Shoot culture establishment: Nodes (2-3 cm) were vertically inoculated in Murashige and Skoog's (MS) medium[15] fortified with sucrose (SRL, India) (3%) and plant growth regulators (PGRs; SRL, India). Cytokinins (2-10 µM) viz. N^6-benzyladenine (BA) and kinetin (Kn) and auxin viz. indole-3-acetic acid (0.5-2 µM), (IAA), were used individually and in combinations. The pH of the medium was adjusted at 5.80 using NaOH/HCl (1 N) and agar (SRL, India) (0.8%) was used as the solidifying agent. Media were sterilized at 121 ºC (15 psi) for 30 min. All the cultures were maintained at 26±2 ºC under 16/8 h (dark/light) photoperiod at 40 µmol m^{-2} s^{-1} provided by cool-white fluorescent lights (Philips India Ltd., India).

HPTLC fingerprint: Eight weeks old *in vitro* shoots from optimized medium were harvested, washed and dried in oven (40 ºC). 1 g powdered shoots were extracted using hexane, ethyl acetate and methanol (AR grade, SRL, Mumbai, India) by earlier optimized method[17]. 10 µl of all the extracts were applied as a band on precoated Silica gel G60 F_{254} plates (0.2 mm thick, E. Merck Ltd., Mumbai, India) with the help of Linomat V sample applicator (CAMAG, Muttern, Switzerland).

The plate was developed in CAMAG twin through glass chamber containing toluene:methanol (8.7:1.3 v/v) as a mobile phase. After development, the plate was dried and scanned at 254 nm, 366 nm and 525 nm (after derivatization using anisaldehyde-sulfuric acid reagent) in CAMAG TLC scanner 3 linked to winCATS software.

Rooting and hardening: Eight weeks old shoots (>3 cm) were utilized for rooting and for *in vitro* rooting the excised shoots were dipped in sterile distilled water followed by bavistin (0.01%) solution (1 min each). They were transferred to full and half strength MS medium (liquid/static) fortified with sucrose (1%) and 2-10 µM of indole-3-butyric acid (IBA; SRL, India). For *ex vitro* rooting, the excised shoots were dipped for 1 min in sterile distilled water and bavistin (0.01%) solution followed by pulse treatment using IBA (8 µM) for 1 min. 5 ml of ½MS basal medium with sucrose (1%) was added to substrates (cocopeat, vermiculite, soil, sand) prior to inoculation of shoots.

All the rooted shoots were transferred to cups containing cocopeat:sand (1:1) for hardening under lab conditions. These cups were covered with polythene bag to maintain humidity and irrigated with sterile distilled water every alternate day. After four weeks, the plants were transferred to the greenhouse for acclimatization and hardening.

Statistical analysis: All the experiments were repeated twice with ten replicates. Means were analyzed using ANOVA (α=0.05) and significant means were further analyzed by Tukey's test using GraphPad Prism 6.01.

Table 1
Effect of plant growth regulators on shoot regeneration from *E. alba* (Eight weeks)

PGRs (µM)			No. of shoots/explant* (Mean±SE)	Response (%)
BA	Kn	IAA		
0	0	0	0.70±0.20 f	60
5	0	0	1.70±0.14 def	100
10	0	0	1.90±0.22 def	100
15	0	0	1.50±0.16 ef	100
20	0	0	1.40±0.15 ef	100
0	5	0	1.30±0.14 ef	100
0	10	0	2.20±0.13 def	100
0	15	0	1.80±0.13 def	100
0	20	0	1.50±0.16 ef	100
5	5	0	0 f	0
10	10	0	0 f	0
15	15	0	0 f	0
20	20	0	1.00±0.14 f	90
5	0	0.5	1.90±0.36 def	80
5	0	1	7.20±0.63 b	100
5	0	2	1.20±0.39 f	60
10	0	0.5	2.0±0.35 def	100
10	0	1	18.40±0.84 a	100
10	0	2	1.90±0.41 def	70
15	0	0.5	1.90±0.41 def	70
15	0	1	7.90±0.61 b	100
15	0	2	0.90±0.32 f	40
20	0	0.5	1.50±0.41 ef	70
20	0	1	5.80±1.25 bcde	90
20	0	2	1.0±0.32 f	40
0	5	0.5	1.40±0.15 ef	100
0	5	1	1.40±0.21 ef	90
0	5	2	1.40±0.21 ef	90
0	10	0.5	2.00±0.24 def	100
0	10	1	4.80±0.68 cde	100
0	10	2	1.40±0.29 ef	80
0	15	0.5	1.90±0.50 def	60
0	15	1	4.00±0.35 cde	100
0	15	2	1.70±0.38 def	70
0	20	0.5	1.30±0.28 ef	80
0	20	1	3.60±0.38 cde	100
0	20	2	1.00±0.40 f	40

*Means (n = 10) followed by same letter are not significantly different ($p \leq 0.05$) using Tukey's test

Results and Discussion

Shoot regeneration: Nodes of *E. alba* when inoculated on basal MS medium induced very less number of shoots. Further fortifying the basal medium with BA, Kn and IAA individually increased the response but failed to induce multiple shoots. Similarly, nodes of *Hemidesmus indicus* failed to induce multiple shoots when these PGRs were used individually[17] whereas earlier report in *E. alba* reported multiple shoots in BA containing medium[9]. After that when synergistic effect of cytokinin and auxin was used, multiple shoot formation was recorded and optimum 18.40±0.84 shoots were formed in medium comprising of BA (10 µM) and IAA (1 µM) (Table 1).

In this combination, bud break from both the nodes was observed at the end of first week. Both the buds elongated and formed shoots during second week, further elongation as well as branching was observed during third and fourth week (Fig. 1a). At the end of fourth week, the cluster was transferred to fresh medium which facilitated shoot elongation and multiplication which continued till eight weeks and healthy elongated shoots were observed at the end of eight weeks (Fig. 1b). Combination of BA and IAA was reported for regeneration in nodal explants of *Kaempferia galanga*[16] and *Celastrus paniculatus*[20].

The shoot number observed in the present study was comparatively higher than documented in earlier studies for the same plant[3,21]. Replacing BA with Kn in combination with IAA evoked less response as compared to BA+IAA fortified medium, which is also reported for *H. indicus*[17] and *Stevia rebaudiana*[27].

HPTLC fingerprint: *In vitro* shoots from optimized media were assessed for their biosynthetic potential and thus extracts were prepared for *in vivo* and *in vitro* shoots. All the extracts were separated on TLC plates and observed under 254 nm (Figs. 2a, 3a, 4a), 366 nm (Figs. 2b, 3b, 4b) and white light after derivatization (Figs. 2c, 3c, 4c). It was observed that almost similar profiles can be seen for *in vivo* and *in vitro* shoots in all three extracts. But when densitometric scanning was done, variation in peak number and their areas between the samples was detected (Figs. 2d-i; 3d-i; 4d-i). This may be due to PGRs of the medium which is known to affect the metabolite synthesis[6,11].

Variation in HPTLC fingerprint due to effect of PGRs is reported for shoot cultures of *H. indicus*[17]. Similarly, this method has been used for assessment of biosynthetic potential of *in vitro* cultures of medicinal plants like *Celastrus paniculatus*[12] and *Asparagus adscendens*[13].

***In vitro* and *ex vitro* rooting and hardening:** Eight weeks old shoots failed to induce roots when placed in full strength liquid MS basal medium, whereas reducing the strength to half formed rooting in shoots. IBA has been used for rooting in many plant species[9,18] and similarly rooting was observed when medium was fortified with different concentrations of IBA. Amongst all the combinations tried, optimum 40.50±1.38 roots were recorded in ½MS liquid medium containing IBA (8 µM) (Fig. 1c).

Table 2
Effect of medium strength and IBA concentrations on rooting of *E. alba* shoots (Four weeks)

Liquid medium				
IBA (µM)	**Half strength**		**Full strength**	
	No. of roots/shoot* (Mean±SE)	**% Response**	**No. of roots/shoot* (Mean±SE)**	**% Response**
0	3.20±0.7 f	70	0 c	0
2	6.40±0.32 de	100	0 c	0
4	21.60±0.49 c	100	0 c	0
6	22.50±0.62 c	100	7.10±0.41 b	100
8	40.50±1.38 a	100	18.70±0.62 a	100
10	18.00±0.65 c	100	6.60±1.78 b	60
Static medium				
IBA (µM)	**Half strength**		**Full strength**	
	No. of roots/shoot* (Mean±SE)	**% Response**	**No. of roots/shoot* (Mean±SE)**	**% Response**
0	3.90±0.94ef	70	0c	0
2	17.50±2.81 c	80	0 c	0
4	18.50±2.01 c	90	4.00±0.63 bc	100
6	29.10±3.15 b	90	4.10±0.91 bc	70
8	15.70±2.55 c	80	22.48±2.48 a	90
10	14.00±2.34 cd	80	0 c	0

*Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) using Tukey's test

Figure 1: Micropropagation of *Eclipta alba* in MS medium fortified with BA (10 µM) and IAA (1 µM)- (a) multiple shoot at the end of four weeks, (b) elongated healthy shoots after eight weeks, (c) rooting in ½MS liquid medium fortified with sucrose (1%) and IBA (8 µM), (d) hardening of plantlets in cocopeat:sand (1:1) under lab condition, (e) acclimatized plant under greenhouse condition and (f) hardened plant in garden soil at flowering stage after 3 months

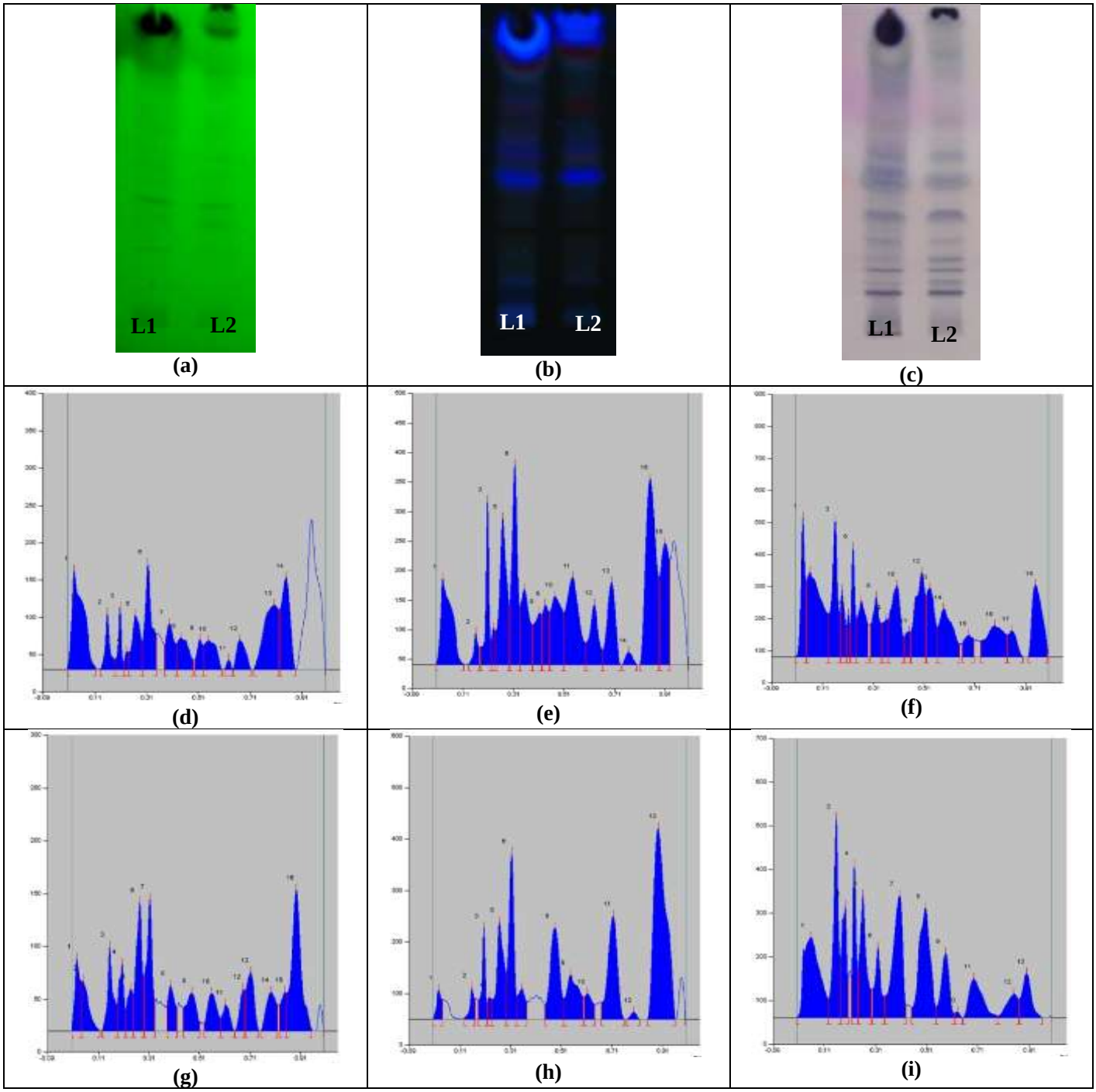

Figure 2: HPTLC fingerprinting of hexane extracts- (a-c) TLC plates containing hexane extracts of *in vivo* (L1) and *in vitro* (L2) shoots at (a) 254 nm, (b) 366 nm and (c) white light, (d-i) densitometric scanning of plates and respective peaks of hexane extracts in which (d-f) *in vivo* and (g-i) *in vitro* shoots after scanning at (d and g) 254 nm, (e and h) 366 nm and (f and i) 525 nm

The observations also revealed that addition of agar in both the strengths of the medium failed to enhance the number of roots. ½MS medium evoked better response as compared to full strength and this is in line with earlier reports on *E. alba*[24,25]. *Ex vitro* rooting was done using different substrates and amongst all the substrates tried, optimum 10.40±1.18 roots (90% response) were formed in vermiculite (Fig. 3).

This method has advantages as it combines rooting and hardening stage and also the roots developed through this technique ensure better hardening and survival of plantlets[19,31].

The plantlets were transferred to the cocopeat:sand (1:1) in which shoots survived better then vermiculite. They were covered with polythene bag, watered every alternate day for hardening under laboratory conditions for four weeks (Fig. 1d). The cups were transferred to greenhouse for further hardening and acclimatizing for four weeks in the same

substrate (Fig. 1e) followed by transferring into garden soil (Fig. 1f).

Conclusion

In this study, an efficient protocol for micropropagation of *E. alba* through nodal explant has been reported and thus this can be used for mass propagation. These shoots have potency to synthesize similar metabolites as mother plant and hence utilized as an alternative to wild plant for extraction of metabolites. Further work will be directed for quantification of metabolites in *in vivo* and *in vitro* shoot as well as elicitation of marker compounds.

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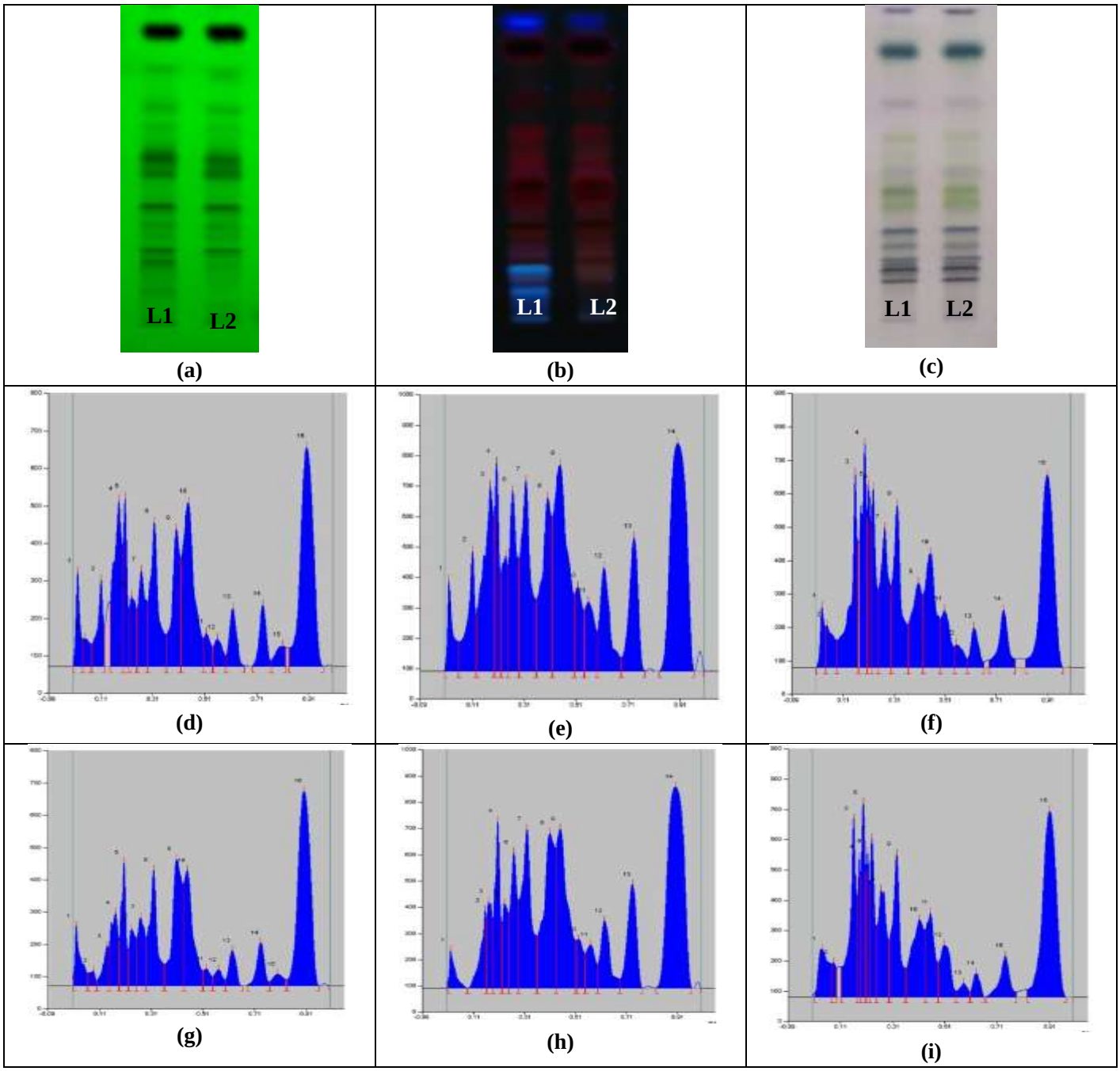

Figure 3: HPTLC fingerprinting of ethyl acetate extracts- (a-c) TLC plates containing ethyl acetate extracts of *in vivo* (L1) and *in vitro* (L2) shoots at (a) 254 nm, (b) 366 nm and (c) white light, (d-i) densitometric scanning of plates and respective peaks of ethyl acetate extracts in which (d-f) *in vivo* and (g-i) *in vitro* shoots after scanning at (d and g) 254 nm, (e and h) 366 nm and (f and i) 525 nm

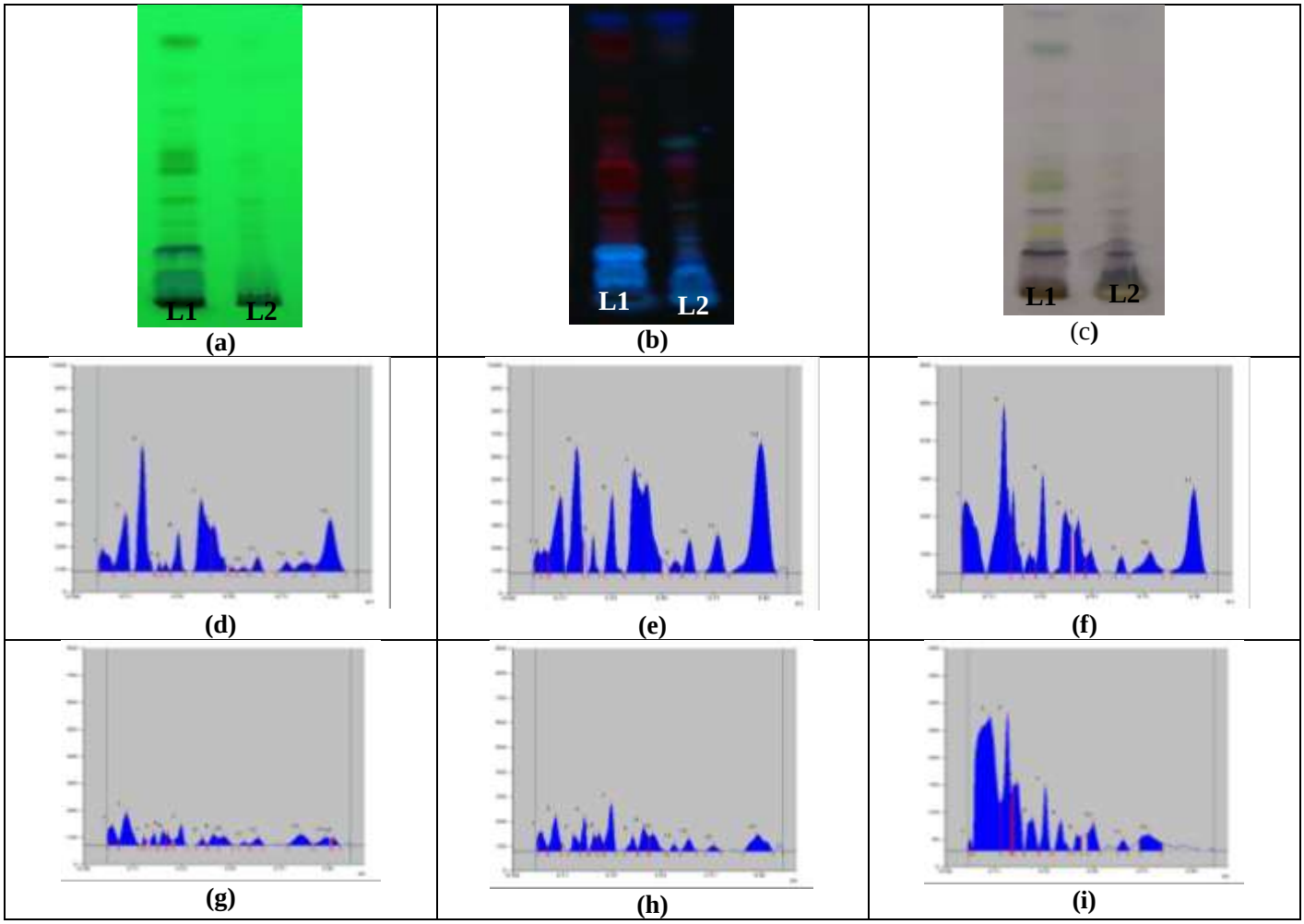

Figure 4: HPTLC fingerprinting of methanol extracts- (a-c) TLC plates containing methanol extracts of *in vivo* (L1) and *in vitro* (L2) shoots at (a) 254 nm, (b) 366 nm and (c) white light, (d-i) densitometric scanning of plates and respective peaks of methanol extracts in which (d-f) *in vivo* and (g-i) *in vitro* shoots after scanning at (d and g) 254 nm, (e and h) 366 nm and (f and i) 525 nm

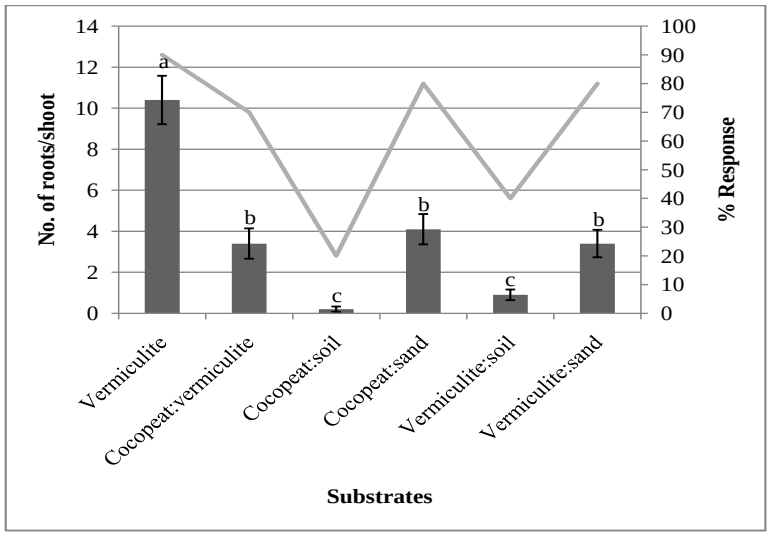

Figure 5: Effect of natural planting substrates on *ex vitro* rooting of *E. alba* shoots. Each bar represents mean values (n = 10), error bar as standard error and line represents the % response. Means followed by same letter are not significantly different ($p \leq 0.05$) using Tukey's test

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