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Original article

Antimicrobial activities of *Acinos rotundifolius* Pers. from extracts of hypocotyl and cotyledon node induced calli

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Summary. *In vitro* callus propagation is extremely important both for generation of a plant's secondary metabolites and for increasing production of these metabolites. This study reports a protocol for callus induction for *Acinos rotundifolius* Pers. hypocotyl and cotyledon node explants obtained from 10 days old *in vitro* grown plantlets. The calli obtained in this way are also examined for their antimicrobial activities. Callus induction was noted on all cotyledon node and hypocotyl explants. For cotyledon node explants the most productive environment in terms of callus weight was MS medium containing 0.04 mg/l TDZ + 0.2 mg/l IBA. Increase of the TDZ concentration resulted in decrease of the callus weight. For hypocotyl explants maximum callus induction in terms of callus weight was noted on MS medium containing 1 mg/l BAP + 0.5 mg/l 2,4-D. The increase of BAP concentration generally resulted in decreased callus weight. Irrespective of the source of calli, crude extracts of *A. rotundifolius* Pers were found very effective for inhibition of *Staphylococcus aureus* COWAN 1 and *Bacillus subtilis* var. niger ATCC 10. The inhibition zone diameter determined by agar diffusion methodology for each extract showed more activity against *Bacillus subtilis* var. niger ATCC 10 compared to *Staphylococcus aureus* COWAN 1. No antimicrobial activity was observed with methanol extracts. There is need to evaluate extracts against more types of bacteria to broaden the scope of *A. rotundifolius* Pers in pharmaceutical practice.

Key words: *Acinos rotundifolius* Pers, callus, antimicrobial activity

Introduction

The number of species, especially endemic species, that are distributed over a country and the variety of vegetation types a country owns are good measures of the floristic richness of a country. Turkey has suitable climate and soil characteristics to grow many medicinal plants that feature a whole continent (1). About 12000 vascular plant taxons are grown in Turkey. Approximately 3000 of them are endemic (2). Among these plants the Lamiaceae family is of great importance in terms of endemism and pharmaceutical characteristics. Lamiaceae is a family of flowering plants showing cosmopolitan distribution that grow in almost all habitat types and at all altitudes with spread to all parts of the world with a significantly high availability in the vegetation of the Mediterranean region (3-5). These plants are annual or perennial and usually herbaceous. Some species grow in form of shrubs or trees and are rarely found as climbers (6). All organs of the plants belonging to this family contain volatile and aromatic compounds that are used in the pharmaceutical and perfume industry. They are also used as spice and tea for their aroma and flavoring. Plant-based chemicals are derived from plants grown *in vivo* for a long time, but the general trend nowadays is the utilization of various *in vitro* culture techniques for production of these secondary metabolites (7). The provision of *in vitro* callus formation has a great importance especially in the production of secondary metabolites synthesized by plants that have effective antimicrobial activity. Therefore, this study intended to develop a protocol for callus formation in *Acinos rotundifolius* Pers. by using cotyledon node and hypocotyl

explants and to test the crude callus extracts for their antimicrobial activity that may have a greater application in treatment of many infectious diseases in future.

Materials and Methods

The seeds of *Acinos rotundifolius* Pers. were obtained from the Department of Biology, Faculty of Science and Art, Bitlis Eren University, Bitlis, Turkey. Voucher specimens of these plants are deposited at the Herbarium of the Faculty of Science, Firat University, Elazig, Turkey. The seeds were treated with 100% commercial bleach (5% NaOCl Ace, Turkey) for 20 min. followed by 3 × 3 min. rinsing with sterilized distilled water. These were cultured on agar solidified MS medium (8) contained in Petri dishes (100 ×10 mm) supplemented with 3% sucrose to sprout them under 16/8 h dark/light photoperiod (35µmol $m^{-2}s^{-1}$) in Aralab versatile growth chamber at 24 ± 1°C. Both cotyledon node and hypocotyl explants were obtained from 10 days old young seedlings. Cotyledon node explants were cultured on MS medium containing 0.04, 0.08, 0.12, 0.16, 0.2, 0.24, 0.28, 0.32 mg/l TDZ and 0.2 mg/l IBA (8 combinations). Hypocotyl explants were cultured on MS medium containing 1, 2, 3, 4, 5, 6, 7, 8 mg/l BAP and 0.5 mg/l 2,4-D (8 combinations) supplemented with 3% (w/v) sucrose and 0.65% (w/v) plant agar (Duchefa). All media were autoclaved for 20 min. at 121°C and 1.4 kg cm^{-2} pressure. The pH of all media was adjusted to 5.7± 0.1 with 1N NaOH or 1 N HCl. Each treatment contained 30 explants that were divided into three equally distributed replications.

Preparation of extracts

The calli obtained from each treatment were dried at 72°C for 72 hours and ground with Waring blender. Thereafter, extractions were made using soxhlet extractor with hot water (control), acetone, hexane and methanol as solvent for 12 h. The solvents used in the study were removed with the help of a rotary vacuum evaporator at 35°C. After carefully determining the percentage yield of each extract, the three extracts were used at a concentration of 12.5 ng/µl after filter sterilization with 0.2 Micron (Whatman 67802502 Polyethersulfone Puradisc 25 Syringe Filter). The extracts were maintained at 4°C for 15 d.

Bacterial strains

Antimicrobial activities for *Staphylococcus aureus* COWAN 1 and *Bacillus subtilis* var. niger ATCC 10 strains of bacteria were studied.

Study of antimicrobial activity

Modified agar well diffusion method was used to study antimicrobial activities of the plant extracts following Vlietinck et al. (9). Both bacterial strains were cultured at 28°C for 24 h using Mueller Hinton broth (MHB, Difco). Bacterial inoculum was well spread after pipetting onto the Mueller Hinton agar medium in 100×10 mm Petri dishes. Thereafter, 12 mm diameter four wells, were made in each quadrant of Petri dish using a sterilized cork borer and each was filled aseptically with 0.1 ml of plant extract. After allowing diffusion of extracts into agar for 1 h at 4°C, the inverted Petri dishes were incubated at 38°C for 24 h for multiplication of bacteria. Thereafter inhibition zone diameters were measured in mm. Tests were performed in triplicate.

Statistical analysis

Each treatment used 30 explants divided into 3 replications. Callus induction observations were recorded after 28 days. Experimental values for each parameter were compared using One Way Anova of IBM SPSS 22 for Windows. Standard error was computed using descriptive statistics function. Means were compared using Tukey's b test. All values expressed as 0.00 were not subjected to statistical analysis.

Results

Callus induction on MS media containing TDZ + IBA

Callus induction was noted for all combinations of TDZ + IBA on cotyledon node explants (Table 1

Table 1. The effects of TDZ + IBA concentrations on callus induction on cotyledon node explants.

Plant growth regulator concentrations and combinations		Rate of callus formation (%)	Callus weight (mg)*
TDZ (mg/l)	IBA (mg/l)		
0.04	0.20	100.00 ± 0.00	463.40 ± 1.66a
0.08	0.20	100.00 ± 0.00	446.20 ± 2.54b
0.12	0.20	100.00 ± 0.00	435.90 ± 1.57c
0.16	0.20	100.00 ± 0.00	286.30 ± 1.65d
0.2	0.20	100.00 ± 0.00	274.60 ± 0.07e
0.24	0.20	. 100.00 ± 0.00	249.80 ± 1.36f
0.28	0.20	100.00 ± 0.00	232.51 ± 0.70g
0.32	0.20	100.00 ± 0.00	216.27 ± 0.49h
Control (MS medium)		0.00 ± 0.00	0 ± 0.00

**All values in the column shown by different small letters are statisitically significant at 0.05 level ± standard error*
Increasing concentrations of TDZ resulted in reduction of callus weight. Use of 0.32 mg/l TDZ resulted in a minimum callus induction that constitutes 53% reduction in maximum callus weight.

– Figure 1). However, induced callus weight varied depending on the concentration and combination of TDZ +IBA. No callus induction was noted, when the explants were cultured on MS medium without growth regulators (control). The results showed that the callus weight ranged from 216.27 ± 0.49 mg to 463.40 ± 1.66 mg (Table 1). The most productive environment in terms of callus weight was the MS medium containing 0.04 mg/l TDZ + 0.2 mg/l IBA with 463.40 ± 1.66 mg callus weight. The increase of TDZ concentration resulted in reduced callus weight. Minimum callus induction was noted with 0.32 mg/l TDZ + 0.20 mg/l IBA (216.27 ± 0.49 mg callus weight).

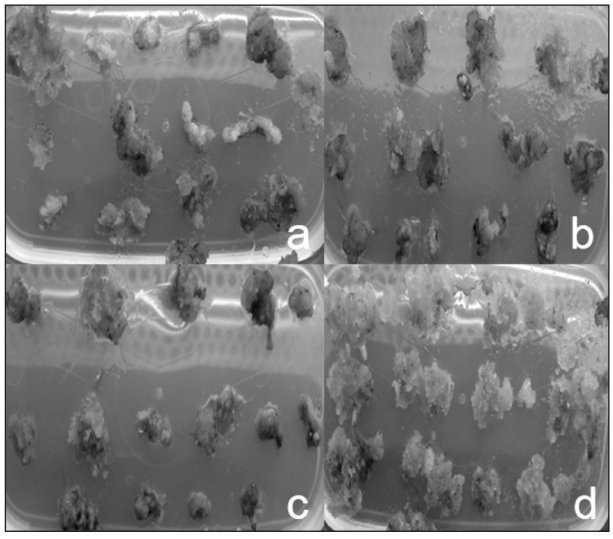

Figure 1. Callus induction on hypocotyl explant of Acinos rotundifolius under in vitro conditions (a). Calli from cotyledon node explants on MS medium containing 0.32 mg/l TDZ + 0.2 mg/l IBA(b). Calli from cotyledon node explants on MS medium containing 0.08 mg/l TDZ + 0.2 mg/l IBA (c). Calli from cotyledon node explants on MS medium containing 0.04 mg/l TDZ + 0.2 mg/l IBA (d). Calli from hypocotyl explants on MS medium containing 1 mg/l BAP + 0.5 mg/l 2,4-D.

Callus induction on MS medium containing BAP + 2,4-D

Callus formed in all our hypocotyl explants (100%) with all combinations of BAP + 2,4-D (Table 2). However the callus weight varied significantly depending on the BAP concentrations of the BAP + 2,4-D combinations. MS medium without growth regulators failed to induce any callus formation (control). The heaviest calli (479.28 ± 0.96 mg) were induced on MS medium containing 1 mg/l BAP + 0.5 mg/l 2,4-D (Table 2). The increase of BAP concentration generally resulted in reduced callus weight. But MS medium containing 4 mg/l BAP + 0.5 mg/l 2,4-D generated an exception with 395.48 ± 2.03 mg callus weight, compared to 361.86 ± 0.72 mg callus obtained with 3 mg/l BAP + 0.5 mg/l 2,4-D. Minimum callus weights of 201.06 ± 1.80 mg and 196.37 ± 1.05 mg was noted on MS medium containing 7 and 8 mg/l BAP + 0.5 mg/l 2,4-D respectively.

Table 2. The effect of BAP and 2,4-D concentrations on callus induction on hypocotyl explants.

Plant growth regulator concentrations and combinations		Rate of callus formation (%)	Callus weight (mg)*
BAP (mg/l)	2,4-D (mg/l)		
1.00	0.50	100.00 ± 0.00	479.28 ± 0.96a
2.00	0.50	100.00 ± 0.00	452.18 ± 1.26b
3.00	0.50	100.00 ± 0.00	361.86 ± 0.72d
4.00	0.50	100.00 ± 0.00	395.48 ± 2.03c
5.00	0.50	100.00 ± 0.00	303.16 ± 1.63e
6.00	0.50	100.00 ± 0.00	252.09 ± 0.47f
7.00	0.50	100.00 ± 0.00	201.06 ± 1.80g
8.00	0.50	100.00 ± 0.00	196.37 ± 1.05g
Control (MS medium)		0.00 ± 0.00	0 ± 0.00

**All values in the column shown by different small letters are statisitically significant at 0.05 level ± standard error*
Increasing concentrations of BAP resulted in reduction of callus weight. Use of 8.0 mg/l BAP resulted in a minimum callus induction that constitutes 59% reduction in maximum callus weight.

Measurement of antimicrobial activity

Calli of the best growth regulator combinations that induced maximum callus weight (0.04 mg/l TDZ + 0.2 mg/l IBA and 1 mg/l BAP + 0.5 mg/l 2,4-D) were investigated. No antimicrobial activities were noted in methanol extracts and control group (Table 3). The hexane and acetone extracts of *A. rotundifolius* Pers calli exhibited inhibitory action against both bacterial strains.

Discussion

A. rotundifolius Pers is an annual herbaceous plant that contains a large number of volatile and aromatic compounds that are used in the pharmaceutical and perfume industry for a long time. We studied callus induction in juvenile cotyledon node and hypocotyl explants raised from seeds and tested their crude callus extracts for antimicrobial activity.

It is important to know about plant growth regulator concentrations suitable for callus induction to achieve valuable secondary metabolites of high pharmaceutical importance in *A. rotundifolius* Pers. The results of this study clearly indicate the role of different plant growth regulators for callus induction. Similar studies were performed with various species in order to achieve the best hormone combinations for callus induction. Sie et al. (10) found that different combinations of 2,4-D and TDZ on a DKW-based medium resulted in effective callus induction for *Hibiscus sabdariffa*. When we compare the hypocotyl and

Table 3. Effects of extracts from BAP+IBA and TDZ+2,4-D induced calli on anti microbial activity against *S. aureus* and *B. subtilis*

Plant growth regulator combinations			Antimicrobial activity		
	Extract type	Yield (%)	Control	S. aureus	B. subtilis
BAP+IBA	Acetone	1.53	Nil	14	17
	Hexane	0.87	Nil	13	17
	Methanol	13.22	Nil	Nil	Nil
TDZ+2,4-D	Acetone	1.53	Nil	12	15
	Hexane	0.87	Nil	14	18
	Methanol	13.22	Nil	Nil	Nil

Inhibition zone diameters were measured in mm.

cotyledon node explants in terms of callus weights, we can conclude that the hypocotyl explants induced heavier calli. Zhang et al. (11) and Zouzou et al. (12) also pointed out that hypocotyls are more callogenic compared to cotyledon node explants. Previous studies on plants belonging to the family Lamiaceae indicate the use of cytokinin and auxin combinations for callus induction. Lemraski et al. (13) used BA-NAA for callus induction on salvia species. Bolta et al. (14) reported best callus induction on MS medium with 10.47 m.mol NAA and 4.5 m.mol BA-6. Dronne et al. (15) also point out that lavender (*Lavendula officinalis*) could best regenerate on MS medium containing NAA + BA as the callusing medium. Researches have shown that the ability of tissue culture and plant regeneration from callus is related to the genetics and many genes in the nucleus and cytoplasm could control it (16). In studies done in conjunction with tissue culture of ornamental plants, researchers concluded that BA-6-benzylaminopurine and NAA-naphthaleneacetic hormones are the most effective growth regulators used for shoot proliferation and rooting, respectively (17). It seems that the antimicrobial activity against *B. subtilis* was more pronounced as compared to *S. aureus*. It must be mentioned that *B. subtilis* is gram negative and *S. aureus* is gram positive bacteria. The variance in the antimicrobial activity is the result of the different cell wall structures as stated by Essawi and Srour (18). *S. aureus* and *B. subtilis* are often used to test the antimicrobial activity of drugs. Yildirim et al. (19) found that ether, ethanol, and hot water extracts of the leaves of *R. crispus* were active against *S. aureus* and *B. Subtilis* (19). Ulukanli et al. (20) reported that hexane extracts of the aerial parts of *A. rotundifolius* had no growth inhibition against *S. aureus* and *B. subtilis* var *niger* (20). This result is not generally in accordance with that of our study. That may be the consequence of different extraction methods used in two studies.

As a conclusion, our study indicates that BAP+2,4-D combinations used for hypocotyl explants and TDZ+IBA combinations used for cotyledon node explants of *A. rotundifolius* Pers are equally good for callus induction. Calli from either of explants are evaluated positive for their antimicrobial activities against *S. aureus* and *B. subtilis* as examples of gram positive and gram negative bacteria, respectively.

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