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Total Polyphenol Content, *in vitro* Antifungal and Antioxidant Activities of Callus Cultures from *Inula crithmoides*

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This is the first report on the antioxidant and antifungal activities of callus cultures from *Inula crithmoides* L. (Asteraceae). Callus cultures were initiated from leaf sections, on initial culture MS basal medium supplemented with various concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (1-naphthaleneacetic acid) and IBA (indole-3-butyric acid) and a 72% survival was achieved. Significant differences between the various auxins used as phytohormones on callus growth were found. Maximum callusing was noticed on the leaf explants grown on MS basal medium supplemented with 1 mgL^{-1} 2,4-D. Subsequently the antioxidant and antimicrobial activities of the methanol extract from calli were investigated. Antioxidant studies suggested that the methanol extracts of dark-grown and light-grown callus were able to reduce the stable free radical 2,2-diphenyl-1-picrilhydrazyl (DPPH). In the inhibition against lipid peroxidation, extracts of dark-grown callus showed the strongest effect with IC_{50} values better than those of the standards. The methanol extract of callus cultures had significant antifungal activity only against two of the fungi tested: *Alternaria solani* and *Phytophthora cryptogea*. Against all the other tested fungi, the *I. crithmoides* calli extracts showed fungistatic activity.

Keywords: Antifungal activity, Antioxidant activity, *Inula crithmoides*, Total polyphenol content, Callus cultures.

The genus *Inula* (Asteraceae), tribe Inuleae (subtribe Inulinae), is represented in Europe by 19 species and a larger number of endemic subspecies. About 25 species of the genus have been investigated chemically. *I. crithmoides* L. is a hairless perennial shrub growing in seaboard locations [1]. The major secondary metabolites of the genus are sesquiterpene lactones mainly eudesmolides [2–4] and other types of sesquiterpene lactones [2,3], monoterpenes [5], diterpenes [6] and flavonoids of diverse chemical structures [7]. Several pharmacological activities are attributed to these secondary metabolites, including treatment of asthma, dysentery and inflammatory diseases [8].

In a recent paper, we reported the chemical composition and the antioxidant activity (5-lipoxygenase assay) of the essential oil from the aerial parts of *I. crithmoides* collected in central Italy [9]. In fact, in the inhibition of lipid peroxidation our oil was twice more effective than BHT and much more effective than Trolox.

In recent years, multiple drug/chemical resistance in both human and plant pathogenic microorganisms has developed, due to an indiscriminate use of commercial antimicrobial drugs/chemicals commonly used in the treatment of infectious diseases [10]. Plant extracts seem to be an alternative to currently used fungicides to control phytopathogenic fungi, as they are rich source of bioactive chemicals [11], biodegradable in nature, non-pollutant and have no residual or phytotoxic properties. These natural products have the potential to replace the present fungicides [12].

Plant cell cultures are, on the other hand, useful systems for the production of antimicrobial and antioxidant principles [13]. These may be considered a de novo production of the necessary secondary metabolites through cell culture. To the best of our knowledge, antimicrobial and/or antioxidant compound production in cell cultures of *I. crithmoides* has not yet been reported. The aim of this study is to optimize a protocol of callus culture from *I. crithmoides* collected in central Italy and to investigate some biological properties of its extracts.

Initial callus induction occurred directly on the cut surfaces of the explants. Young leaves were the best source of explants. In this experiment, the effect was tested of several plant growth regulators for callus induction, at different concentrations (0.5-2.5 mgL^{-1}). Significant differences between the various auxins used on callus growth were found. The results obtained in callus induction and growth are reported in Tables 1 and 2.

Explants from young leaves produced significantly more callus than those from older leaves (data not shown) and only the former were used for further experiments. Leaf cultures in hormone-free basal medium did not produce any callus, and explants died after a few days of incubation. In order to determine the influence of auxins on callus induction, 2,4-D, IBA and NAA (Plant Growth Regulators = PGRs) were used. No calli were induced in the MS basal medium without the plant growth regulators (Tables 1 and 2) indicating that PGRs are required for callus induction. Maximum callusing was noticed on the leaf explants grown on MS basal medium supplemented with 1 mgL^{-1} 2,4-D (90.2% in light-grown and 100% in dark-grown). Most of the calli were found covering the surface of the explants; they were friable and green when grown in the presence of light, and compact and white in dark-grown callus. They grew well during subsequent subcultures. IBA or NAA at the same concentration as 2,4-D were poor in inducing calli from *I. crithmoides* (40.2% and 48.3% in the presence of light, respectively; 80.2% and 83.3% in the dark, respectively).

Our data are in agreement with the literature [14-16] and showed that 2,4-D was superior to other auxins in callus induction from *I. crithmoides*. In the present work, the best response in terms of

Table 1: Callus induction from leaf explants of *Inula crithmoides* inoculated on MS media supplemented with 1.0 mgL^{-1} of various types of auxins and light-grown.

MS + 1.0 mgL^{-1} auxin	Callus formation	Explant forming callus (%)	Callus morphology
MS	NC	NC	NC
MS +2,4 D	+++	90.2	Green friable
MS + IBA	+	40.2	Whitish friable
MS + NAA	+	48.3	Whitish friable

Legend: NC = no callus formed; + = callus only formed at the edge of the explant; +++ callus covered the surface

Table 2: Callus induction from leaf explants of *Inula crithmoides* inoculated on MS media supplemented with 1.0 mgL^{-1} of various types of auxins and dark-grown.

MS + 1.0 mgL^{-1} auxin	Callus formation	Explant forming callus (%)	Callus morphology
MS	NC	NC	NC
MS + 2,4 D	+++	100	Compact and white
MS + IBA	+	80.2	Compact and white
MS + NAA	+	83.3	Compact and white

Legend: NC = no callus formed; + = callus only formed at the edge of the explant; +++ callus covered the surface

biomass production was obtained using 2,4-D. On the basis of the average of fresh and dry weight, in both cases (light and dark), the highest growth rate was observed between days 20 and 25 (data not shown); we used 20, 30 and 40-day-old calli for the determination of phenolic content and antioxidant and antifungal activities.

Total polyphenol content: In many fruits and vegetables, the antioxidant activity can be attributed to the level of total polyphenols [17]. In this study, the total polyphenol levels were measured in callus cultures obtained in the presence of light and in the dark; a similar trend was exhibited in both cases (Table 3).

Table 3: Polyphenol content of *Inula crithmoides* callus extracts.

Callus age (days)	Light (mg/g DW)	Dark(mg/g DW)
20	4.4 ± 0.38	15.0 ± 1.22
30	14.0 ± 0.95	18.01 ± 1.53
40	28.3 ± 1.90	18.90 ± 1.51

In light-grown callus, polyphenol content increased with age and at 40 days of culture was about 7 and 2 times higher than at 20 and 30 days, respectively. On the contrary, the polyphenol content of extracts obtained from dark–grown callus was initially higher and did not increase during the subsequent 40 days of culture.

One of the reasons for research on various plant cell, tissue or organ cultures is the ability of these cultures to synthesize *in vitro* some of the metabolites that are found in the whole plants [18]. Thus, this becomes an alternative for obtaining products that are difficult to obtain either by conventional methods or whose production is not economically viable.

The radical scavenging activity of callus extracts was evaluated by the DPPH test and reported in Table 4. The antioxidant activity appeared to be influenced by time in culture, and presence/absence of light. In light-grown callus, antioxidant activity increased with callus age attaining the maximum value at 40 days (EC_{50} = 1.08) with an EC_{50} 10 times greater than that of the standard (BHT=EC_{50} = 0.087). On the contrary, in calli grown in the dark, the EC_{50} value is comparable with that of BHT (callus extracts, EC_{50}=0.090; BHT, EC_{50} = 0.087, respectively).

The curve (data not shown) for the radical scavenging activity of *I. crithmoides* callus extract was found to be dose dependent. Scavenging of DPPH radicals was found to increase as the concentration of the callus extract increased. The antioxidative property of this extract can be attributed to the presence of phenolic compounds [19]. The correlation between total phenolic compounds and DPPH scavenging activity was significantly high (r^2 = 0.9215; p<0.01) (data not shown). The antioxidant activity of the extracts obtained from dark-grown callus indicates that they have a protective effect against ROS and can, therefore, be used as a natural preservative ingredient in the food and pharmaceutical industries.

The results obtained with the lipoxygenase assay showed that all the extracts tested exhibited a notable inhibitory effect on lipid peroxidation (Table 4).

Table 4: Antioxidant activities of *Inula crithmoides* callus extracts.

Callus age (days)	DPPH test EC_{50} (mg/mL)		Lipoxygenase assay IC_{50} (µg/mL)	
	light	dark	light	dark
20	10.2 ±0.9	5.4 ±0.4	12.9±1.1	2.7± 0.2
30	4.0 ±0.4	1.0 ±0.8	5.5 ± 0.5	1.4 ± 0.1
40	1.1 ±0.9	0.09 ±0.009	4.0 ± 0.4	0.05 ± 0.004
BHT	0.087 ± 0.012		3.8 ± 1.3	
Trolox	0.007 ± 0.0009		11.9 ± 1.2	
Ascorbic Acid	0.110 ± 0.007		18.6 ± 1.3	

In light-grown callus, antioxidant activity increased with time in culture and the largest increase occurred between days 30 and 40; the highest antioxidant activity was detected at 40 days with an IC_{50} comparable with that of BHT, showing an even better IC_{50} than those of Trolox and ascorbic acid. Even better values for dark-grown calli were obtained. In fact, in dark-grown calli, at day 20 in culture, antioxidant activity was significantly higher than in calli of the same age grown in light. After a slight increase at 30 days, it further increased, and at 40 days it was significantly higher, with an IC_{50} value better than that of BHT. The antioxidant activity observed in *in vitro* cultured callus is particularly important, since the production of active principles can be provided throughout the year.

Antifungal activity: Plant extracts seem to be an alternative to currently used fungicides to control phytopathogenic fungi [20]. In our work we evaluated the antifungal activity of the organic extracts obtained from callus cultures because, to the best our knowledge, no data on the antifungal activity of callus culture extracts against phytopathogenic fungi are reported in the literature.

The antifungal activities of the extracts were qualitatively and quantitatively assessed by evaluating the presence of inhibition zones, estimation of zone diameters, and determination of MIC values. As shown in Tables 5 and 6, callus extract did not show a good activity against any of the *Fusarium* species tested; only fungistatic activity was observed. However, the extracts obtained from light-grown callus were completely active against *Alternaria solani* and *Phytophthora cryptogea*. At 3200 ppm, a fungicidal activity was detected; for dark-grown callus this activity was demonstrated at 1600 ppm. For these fungi, minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values were also calculated and reported in Table 7.

On the basis of these results, it is possible to conclude that the callus extracts possess a wide antifungal spectrum against *A. solani* and *P. cryptogea*. In fact organic callus extracts may be used as biofungicides against *A. solani* and *P. cryptogea*, but not against *Fusarium* species, in respect of which the extracts showed only fungistatic activity. However, the efficiency of such extracts depends greatly upon the resistance offered by the different fungal species. Further studies are required to investigate the qualitative characterization of extracts and their *in vivo* efficacy [21,22].

Table 5: Antifungal activity of *Inula crithmoides* callus light-grown.

	Inhibition (%)						
Phytopathogenic fungi	Nystatin 50 ppm	extracts 200 ppm	extracts 400 ppm	extracts 800 ppm	extracts 1600 ppm	extracts 3200 ppm	extracts 6400 ppm
F. poae	100b	31.0	45.2	49.1	53.5	84.5	100c
F. graminearum	100b	38.2	67.1	73.7	78.5	85.0	100c
F. semitectum	100b	42.5	47.2	52.5	64.0	83.5	100c
F. oxysporum	100b	39.3	46.8	53.2	57.2	78.0	100c
F. culmorum	100b	48.0	62.5	62.5	62.5	82.3	100c
F. avenaceum	100b	29.0	40.1	52.0	58.0	77.5	100c
A. solani	100b	47.5	84.2	100c	100b	100b	100b
P. cryptogea	100b	58.0	92.2	100c	100b	100b	100b

b=fungicidal; c = fungistatic

Table 6: Antifungal activity of *Inula crithmoides* callus dark-grown.

	Inhibition (%)						
Phytopathogenic fungi	Nystatin 50 ppm	extracts 200 ppm	extracts 400 ppm	extracts 800 ppm	extracts 1600 ppm	extracts 3200 ppm	extracts 6400 ppm
F. poae	100b	30.2	45.4	49.1	54.7	83.5	100c
F. graminearum	100b	35.2	66.1	73.7	77.5	85.0	100c
F. semitectum	100b	40.5	46.0	52.0	64.5	82.0	100c
F. oxysporum	100b	36.3	46.4	54.0	57.2	78.1	100c
F. culmorum	100b	43.4	63.0	63.0	63.0	82.0	100c
F. avenaceum	100b	28.4	37.5	52.0	52.0	77.0	100c
A. solani	100b	45.5	64.2	82.3	100c	100b	100b
P. cryptogea	100b	56.6	70.0	92.0	100c	100b	100b

b =fungicidal ; c = fungistatic

Table 7: MIC and MFC of *Inula crithmoides* callus extracts.

Phytopathogenic fungi	MIC values (µg/mL)		MFC values (µg/mL)	
	Callus Light-grown	Callus dark-grown	Callus light-grown	Callus dark-grown
A. solani	1200	500	2000	900
P. cryptogea	1300	600	2500	1000

In conclusion, the extracts obtained by callus culture can be used as antifungal agents in the management of plant diseases and preservation and/or extension of the shelf-life of raw and processed foods.

Experimental

Plant material: Aerial parts of *Inula crithmoides* were collected during the flowering period (August) at Fano (PU), in the area restricted to coastal habitats, and authenticated by Doctor Laura Giamperi, Botanical Institute, University of Urbino. Voucher specimens of these plants have been deposited at the Herbarium of the Botanical Garden of the University of Urbino (GS 200).

Callus cultures: Callus cultures were initiated from leaves washed in 3% Teepol detergent solution, rinsed under running water, sterilized with 0.1%, w/v, $HgCl_2$, and then rinsed 3-4 times with sterile distilled water. Leaves were excised aseptically and cultured on MS basal medium [23] supplemented with 3% sucrose, 0.8% agar and various concentrations of 2,4-D (2,4-dichlorophenoxy-acetic acid), NAA (1-naphthaleneacetic acid) and IBA (indole-3-butyric acid) at pH 5.7. Explants were incubated in a growth chamber at 25°C ± 2°C either in the dark or in the light (cool white fluorescent light at 50 µmol $m^{-2}s^{-1}$ under a 16 h photoperiod). Callus produced from leaves was separated from the initial segments and subcultured at 4-week-intervals on the same medium. Callus from the fifth subculture was utilized for all the experiments. Callus growth was evaluated by measuring dry and fresh weight every 5 days throughout a 40-day culture period. The proliferated calli of each treatment were weighed and recorded after one month of sub-culture.

Plant extraction procedure: All the samples, i.e., 10, 20, 30 or 40 day-old callus, were homogenized in a mortar with 2-5 vol of methanol and centrifuged at 15000 *g* for 10 min at room temperature. The supernatants were either immediately used for analyses or stored at -80°C until use.

Determination of total polyphenol content: Total content of polyphenolic compounds was determined by the Prussian Blue method described by Hagerman and Butler [24, 25], with slight modifications. Quercetin (Sigma Chemical Co., St. Louis, MO) was used as standard.

Antioxidant activities

DPPH test: Radical scavenging activity was determined by a spectrophotometric method based on the reduction of an ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) [25, 26]. Tests were carried out in triplicate. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), BHT (butylated hydroxytoluene) and ascorbic acid were used as positive controls and purchased from Sigma. The EC_{50} values, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, were calculated from the results.

Lipoxygenase test: Inhibition of lipid peroxide formation was evaluated by the 5-lipoxygenase assay in test samples and in positive controls. The activity of the enzyme was assayed spectrophotometrically according to Holman. This method was modified by Sud'ina *et al*. [25, 27]. The IC_{50} values, defined as the amount of antioxidant necessary to inhibit lipid peroxidation by 50%, were calculated from the results.

Antifungal activity

Biological screening: Fungal plant pathogens used in these tests were *Fusarium poae* (Peck) Wollenweber, *F. graminearum* Schwabe, *F. semitectum* Berkeley et Ravenel, *F. oxysporum* Schl., *F. culmorum* (Smith) Saccardo, *F. avenaceum* (Corda: Fries), *Alternaria solani* and *Phytophthora cryptogea*, kindly supplied by the DI.PRO.VAL. (Dipartimento di Protezione e valorizzazione Agroalimentare, università degli studi di Bologna). All of the microorganisms used were maintained in potato dextrose agar (PDA), Sigma) and subcultured every 30 days.

Disc diffusion assay: The phytopathogenic fungi were tested by an agar dilution method [20,28] and extract concentrations of 200, 400, 800, 1600, 3200 and 6400 ppm were tested. The values were expressed in terms of percent inhibition of growth compared with control = 100. The fungicidal activity of the extracts was determined using the technique of Thompson [29]. Negative control was prepared using the same solvent employed to dissolve the calli, and Nystatin (50 ppm) was used as positive control. Each assay was repeated twice.

Statistical analysis: All data are the average of triplicate analyses. The data were recorded as mean ± standard deviation and analysed by SPSS (Version 9.0 for Windows 98, SPSS Inc.).One–way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range test. p values of < 0.05 were regarded as significant and *p* values of < 0.01 very significant.

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