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Short communication

A three-step culture system to increase the xanthone production and antifungal activity of *Hypericum perforatum* subsp. *angustifolium* in vitro roots

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ABSTRACT

Hypericum perforatum is a well-known medicinal plant. Among all secondary metabolites produced by this species, xanthones are very interesting for their antifungal activity. In the present study, with the aim to improve xanthone production and antifungal activity of *H. perforatum* subsp. *angustifolium* (sin. Fröhlich) Borkh in vitro roots, a new methodology consisting of a three-step culture system, has been developed. Regenerated roots of *H. perforatum* were cultured in a three-step culture system: in the first step, to increase biomass, the roots were cultured in half-strength liquid Murashige and Skoog (MS) medium supplemented with 1 mg L^{-1} indole butyric acid (IBA) and 1.5% sucrose.

In the second and third steps, to stimulate secondary metabolism, the roots were cultured with 1.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.215 mg L^{-1} kinetin (KIN), and 0.186 mg L^{-1} 1-naphthalenacetic acid (NAA). In the third step, some of the roots were treated with chitosan. Xanthone production increased 2.7 times following the three-step method. The mean minimal inhibitory concentration (MIC) values were of 36.9, 26.7, and 65 μg mL^{-1}, against *Candida* species, *Cryptococcus neoformans* and dermatophytes, respectively.

A positive correlation between xanthone accumulation and antifungal activity has been shown.

1. Introduction

In the past 30 years there has been a dramatic increase in the incidence of fungal infections [1] and a consequent greater need for effective therapy. However, only a limited range of antifungal agents are available, and some of the most effective agents are toxic. Furthermore, clinical efficacy may be compromised by intrinsic or acquired drug resistance [2].

The increasing resistance of pathogenic fungi to antifungal compounds and the reduced number of available drugs has led to the search for therapeutic alternatives among natural products. In recent years, many studies have been published on the antifungal activity of plant-derived phenolic compounds, including xanthones [3,4]. Several authors have reported the antifungal activity of a broad range of both synthetic and naturally occurring xanthones with respect to *Candida*, *Cryptococcus*, and dermatophytes. In particular, natural and synthetic oxygenated xanthones have been found to be active mainly on dermatophytes [5]. The xanthone α-mangostin, extracted from the fruit of *Garcinia mangostana*, has been shown to inhibit the growth of *Candida albicans* at 1000 μg mL^{-1} [6]. Toxyloxanthone C, isolated from the roots of *Cudrania fruticosa*, has shown antifungal activity against *C. albicans*, with minimal inhibitory concentrations of 25 μg mL^{-1} [7]. Xanthones isolated from the roots of *Hypericum roeperanum* have exhibited antifungal activity against *C. albicans* [8], whereas nothing is known about the activity of extracts from *Hypericum perforatum* roots. However, the content of bioactive compounds extracted from wild plants is not constant and may change according to the developmental stage of the plant, the season, and environmental factors. In vitro cultures are an attractive tool for obtaining extracts rich in bioactive metabolites which are not subject to fluctuation. The potentiality of *H. perforatum* cell cultures to produce xanthones has been already demonstrated by the authors [9].

Moreover, we recently established root cultures of *H. perforatum* subsp. *angustifolium* to obtain extracts with a stable chemical composition [10]. The extracts showed antifungal activity against

Abbreviations: MS, Murashige and Skoog; IBA, indole butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; KIN, kinetin; NAA, 1-naphthalenacetic acid; MIC, minimal inhibitory concentration.

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human pathogenic fungi, and there was a positive linear relationship between antifungal activity and xanthone content in the root extracts. In vitro organ growth and secondary-metabolite production are often governed by different combinations of growth regulators. In light of our previous results, in this study we developed a method to increase the content of bioactive metabolites in the extracts; in particular, we applied a new culture system consisting of three steps which differ in the type and concentration of the growth regulators and by the addition of an elicitor. Herein we report the results of the application of this system in terms of the production of bioactive metabolites and antifungal activity against *Candida* species, *Cryptococcus neoformans* and dermatophytes of *H. perforatum* in vitro roots.

2. Results

To achieve the most suitable culture conditions in terms of biomass and secondary metabolite production, the roots were cultured in a newly developed three-step system. In the first step, the roots, which were cultured in Medium 1, showed a growth index (GI) of 5.4, which corresponded to a 6.4-fold increase in biomass with the respect to the initial inoculum (Fig. 1A). In the second step, although the GI of the roots cultured in Medium 2 (Fig. 1A) was significantly lower ($P < 0.05$) than the GI of the roots cultured in Medium 1, xanthone production was 3.53 times higher (i.e., 12.84 mg g^{-1} DW vs. 3.64 mg g^{-1} DW) (Fig. 1B). The third step, which included the addition of chitosan as an elicitor, led to a further increase in total xanthone content (1.4 times higher than that in the untreated roots) ($P < 0.05$) (Fig. 1B). Xanthone production in both chitosan-treated and untreated roots peaked at day 15 of culture and then gradually decreased (Fig. 1B). Chitosan induced an increase for all of the xanthones (Table 1). The main xanthones accumulated were: 5-methoxy-2-deprenylrheediaxanthone B and paxanthone (Table 1 and Fig. 2). Moreover, the chitosan-treated roots produced 1,7-dihydroxyxanthone (Fig. 2), which was not detected in the untreated roots (Table 1). After the addition of chitosan, no negative effects were observed on root vitality, although it led to a lower growth of biomass (Fig. 1A).

In Table 2, the antifungal activity of roots transferred to Medium 2 and those maintained in Medium 1 is shown. The mean MIC value for roots cultured in Medium 2, compared to the mean MIC value for roots maintained in Medium, 1 was 10 times lower for *C. albicans*, 5.4 times lower for the non-*albicans Candida* species, 5.9 times lower for *C. neoformans* and 5.2 times lower for dermatophytes. In the third step, the addition of chitosan led to an increase of antifungal activity with the mean MIC values of 36.9 μg mL^{-1} for *Candida* spp., 26.7 μg mL^{-1} for *C. neoformans,* and 65 μg mL^{-1} for dermatophytes (Table 3). Moreover, the root extracts showed a low cytotoxicity for both MDCK and HeLa cells (i.e., $CC_{50} > 500$ μg mL^{-1}).

3. Discussion

The increasing occurrence of fungal infections is a major public-health concern, and additional antifungal agents must be developed to successfully control the human fungal pathogens that are resistant to available antifungals. Phytomedicine, which has historically been an important aspect of traditional medicine in non-industrialized countries, is now becoming an integral part of healthcare in industrialized nations.

Plants are the source of thousands of new phytochemicals, and different strategies can be applied to improve the yields of bioactive metabolites in the plant and to obtain standardized extracts. We recently evaluated a biotechnological system, in vitro root cultures of *H. perforatum* subsp. *angustifolium* [10], as an effective method for obtaining qualitatively and quantitatively standardized extracts. To improve the efficiency of this system, we performed new experiments, taking into account that in vitro organ growth and secondary metabolite production are often governed by different combinations of growth regulators and that secondary plant metabolism is generally stimulated when cell growth is slowed [11]. These considerations led us to develop a new three-step culture system. The first step consisted of culturing roots in Medium 1, which contained indole butyric acid (IBA) as auxin, in order to obtain a consistent amount of root biomass. The second step consisted of subculturing roots in Medium 2, which contained 1-naphthalenacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, used to increase secondary metabolite production. Previous studies have shown that NAA stimulated xanthone accumulation in cell suspension cultures of *H. perforatum* [12,13]. In *H. perforatum* subsp. *angustifolium* root cultures, the positive effect of the combined growth regulators (Medium 2) on both xanthone production and antifungal activity is reported for the first time in the present study. Xanthone content in both chitosan-treated and untreated roots peaked at day 15 of culture and then gradually decreased. The decrease in xanthone accumulation could be explained as a deterioration of the xanthones previously produced.

The results demonstrate that the roots transferred to Medium 2 had 3.53 times higher xanthone production and from 5 to 10 times higher antifungal activity against *Candida* spp., *C. neoformans*, and

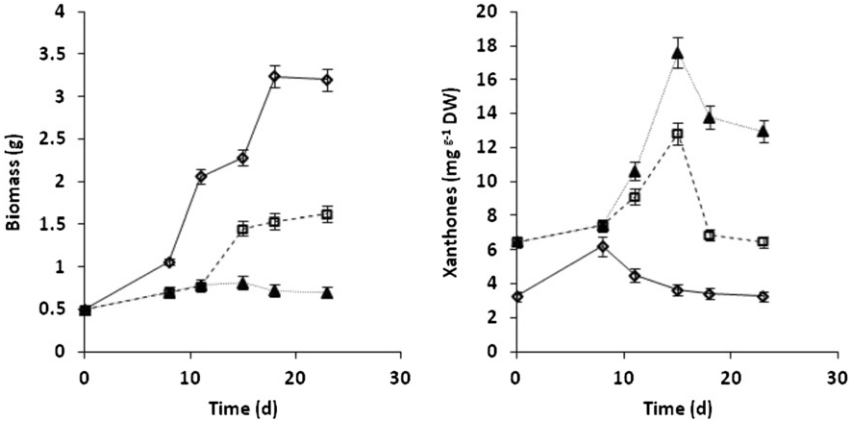

Fig. 1. (A) Growth curves of roots cultured in the Media 1, 2 and 2 supplemented with chitosan during a culture period of 23 days (d). (B) Accumulation of xanthones in roots cultured in the Media 1, 2 and 2 supplemented with chitosan during a culture period of 23 days (d). Results are expressed as means (±SD) of three independent replicates. (◇) Medium 1, (□) Medium 2, ▲ Medium 2 supplemented with chitosan.