Metabolite Production in Callus Culture of Burdock (*Arctium lappa* L.)

THESIS

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Abstract

Burdock (*Arctium lappa* L.) is a biennial plant that is generally regarded as a weed in the U.S. However, in the Amish community, it has been used for treating burns by placing burdock leaves on the wounds after the application of salve. This folk medicine is believed to be effective to speed healing, relieve pain, reduce scars, and prevent infection. More knowledge of the mechanism of burdock treating burns and practical methods for its application is needed before burdock can be recommended as a conventional burn treatment. Few studies have been conducted to determine the basis for burdock treatment for burns. The objectives of this research were to develop callus culture technique for phytochemical production from burdock for eventual medical usage, and analyze these potential medicinal metabolites. The metabolites in burdock we were most interested in were phenolic compounds because of their high content in burdock and their well-known antioxidant bioactivities and related properties which are likely to be useful for treating burns.

The methods we used included plant tissue culture and biochemical analysis. We first developed procedures for burdock callus culture by testing different explant types and culture conditions, with emphasis on the type and concentrations of growth regulators in the culture media. After burdock callus culture was developed, we added different
concentrations of methyl jasmonate and sucrose to the culture media and assessed their
effects on callus growth and phenolic production. To identify and quantify the phenolic
components in burdock callus, we analyzed the phenolic extraction from callus by the
Folin-Cuicaktey assay and reversed-phase HPLC-DAD methods.

Burdock callus could be induced from burdock cotyledon or leaf sections in the dark on
media with 1 mg/L BA and 2 mg/L 2, 4-D, and then maintained over the long term on
media with 1.5 mg/L NAA and 1 mg/L 2, 4-D. Various phenolic compounds were
detected by HPLC and preliminarily identified as hydroxycinnamic acid derivatives
based on their retention times and UV-spectra. Addition of methyl jasmonate up to 1 mM
and 60 g/L and 90 g/L sucrose increased the content of total phenolic and some major
individual phenolic compounds in burdock callus, but decreased callus growth. The
optimum concentrations were 1 mM or 0.1 mM MeJA and 30 g/L sucrose for high total
phenolic production (total phenolic content \times callus relative growth). Our results provide
a foundation for further studies on burdock metabolite production. The callus culture
system can be used as a method for future phytochemical study of burdock and practical
production of burdock medicinal metabolites.
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Chapter 1: Introduction

Burdock

Burdock (Arctium lappa L.) has been used as a medicinal plant and health food for many years. It has been applied as a skin care herb for animals in Eurasia, a traditional herbal medicine in China, and a nutraceutical vegetable in Japan and Korea. Burdock is thought to possess strong antioxidant, bactericidal, and fungicidal activities, which are associated with its medicinal usage. As a health food, burdock is considered to contain antioxidants (Duistermaat, 1996).

The burdock species studied in this research, Arctium lappa, is a member of a small genus within the Asteraceae, with only two other species widely recognized. Burdock is native to Eurasia, and has spread widely to North America and Asia. It is a biennial plant, which germinates in the spring, flowers in the second year from late July to early August, and produces seeds. Reproduction is thought to be mainly through cross-pollination, with the generation of some interspecific hybrids with other Arctium species (Duistermaat, 1996). This branched herb can grow to more than 2 m in height, with a deep taproot. Basal leaves are ovate-cordate, growing up to 80 cm long, with a heart-shaped base and abundant hairs, especially on the lower surface. The solid petiole of the basal leaves and
corymbose inflorescence distinguish *Arctium lappa* from other *Arctium* species. The outer phyllaries have hooked ends that facilitate seed dispersal by animals (including humans) when the seed heads catch on fur or clothing (Gross et al., 1980).

Burdock is a potential resource for medical usage because its roots, stems, and leaves have been found to contain high levels of compounds that are associated with medically useful bioactivity. Among the chemicals extracted and identified from burdock, chlorogenic acid, rutin, caffeic acid, and p-coumaric acid were reported as antioxidants (Lou et al., 2010A). Three lignans, (-)-arctigenin, (-)-matairesinol and (+)-7, 8-didehydroarctigenin, from burdock fruit were shown to have anti-proliferative effects due to apoptosis (Matsumoto et al., 2006). Arctiin and arctigenin are the main lignans in burdock (Liu et al., 2005). Luteolin extracted from burdock has anti-inflammatory activity (Ferracane et al., 2010). Inulin and benzoic acid can prevent bacterial infection (Lou et al., 2010B). Fructooligosaccharide (FOS) has prebiotic effects (Imahori et al., 2010).

Burdock use for burn treatment

In the Amish Community in the U.S., burdock has been used as a folk medicine for treating burns for over 40 years. Burdock leaves about 20 cm in length are harvested, and following removal of the midrib, are laid flat to dry in a warm and dark place for storage. Prior to use, the dried leaves are re-hydrated in boiling water and then placed over the
wound as a bandage following application of a salve containing honey, bees-wax, lanolin and small amounts of plant extracts. According to Amish healers, this treatment prevents bacterial infection, speeds healing, and eases pain when the dressing is changed. Amish healers, and some members of the medical community who work with the Amish community, believe that the burdock leaf wrap plays an essential role in the rapid healing, pain relief, and reduction in scarring that patients experience with this treatment.

Although burdock has been used as an effective burn treatment in the Amish Community, it is not recognized as a conventional treatment for burns in the U.S. One reason is that the profiles of functional compounds in burdock leaves are still unclear, and techniques to produce bioactive metabolites at a repeatable concentration from burdock for burn treatment are unknown. Knowledge of medicinal compound production from burdock and the metabolic profiles of these compounds are needed before burdock can be accepted into medical practice.

Phenolics in burdock might be responsible for burn treatment

Phenolics are widely distributed in plants with over 8000 natural structural variants, which in common possess one or more aromatic rings bearing one or more hydroxyl substituents. Phenolic compounds in plants can be classified as phenolic acids, flavonoids, tannins, stilbenes, lignans, curcuminoids, quinones based on the number of phenolic rings and structural elements. A major class of plant-derived phenolics is
phenolic acids, which comprise two subgroups: hydroxybenzoic acid (aromatic compounds with a one-carbon side chain, C₆-C₁ structure) and hydroxycinnamic acid (aromatic compounds with a three-carbon side chain, C₆-C₃ structure). Phenolics play an important role in plant reproduction, growth, and defense against pathogens, parasites, and predators (Huang et al., 2010). They possess various physiological and pharmacological functions associated to human health, including anti-carcinogenic, anti-inflammatory, anti-aging, skin-protective (Hsu, 2005; Svobodova et al., 2003), neuroprotective, and cardioprotective effects (Boudet, 2007). Their health benefits are thought to be attributed to their antioxidant activity (Balasundram et al., 2006).

Burdock leaves are a rich source of phenolics (Lou et al., 2010B). The properties of plant phenolic compounds as antioxidants (Duh, 1998), and their diverse medical applications including anti-inflammatory activity (Shetty and McCue, 2003) and antimicrobial functions (Shetty and Wahlqvist, 2004), are likely to be responsible for the usefulness of burdock leaves in burn treatments.

Few studies exist on the identification of phenolic compounds of burdock. Two kinds of lignans, arctiin and arctigenin, were extracted and identified from burdock leaves by Liu et al. (2005). Ferracane et al. (2010) identified phenolic compounds in hydro-alcoholic extracts from burdock seeds, roots, and leaves, and determined the antioxidant activity, LC/MS/MS characterization, and total phenolic content from the extracts. Among the phenolic acid reported were caffeic acid, chlorogenic acid, rutin, and cynarin. The authors suggested that burdock can be a good source of antioxidants. However, an extensive
literature search found no research has been dedicated to the production of phenolics from burdock.

Use of plant cell culture to produce phenolics

Producing plant-derived medicinal metabolites from field-grown plants is difficult because variable field environments and cultural practices can lead to inconsistent production of potentially useful metabolites. Field-cultivated, out-crossing burdock plants exhibit considerable phenotypic variation, presumably due to environmental and genetic variation (Gross et al., 1980). It is likely that phenolic compounds of medical interest will also vary with genetics and environmental conditions; therefore, wild, field-grown burdock is unlikely to provide a consistent and reliable source of plant material for phenolic production.

Plant cell culture offers a more reliable method for producing phenolic compounds from burdock. Cell culture is used to grow or maintain plant cells under controlled sterile conditions on a nutrient culture medium. Some advantages of plant cell culture for production of medicinal compounds, as opposed to extracts from field-grown plants include a short production cycle, the ability to produce more consistent and stable products, and the ability to modify and precisely control *in-vitro* conditions to stimulate compound production (Staba, 1997). The first commercialization of plant cell culture used for plant metabolite production was applied to shikonin production by
Lithospermum erythrohizon cells in mid-1980s. Since then, commercial production of plant secondary products from plant cell cultures has successfully been applied to ginseng saponins from Panax ginseng, berberine from Coptis japonica, paclitaxel from Taxus spp., rosmarinic acid from Coleus blumei, and plant polysaccharides from Polianthes tuberosa cultures (Georgiev et al., 2009; Weathers et al., 2010). A commercially significant productivity of tissue cultured materials is needed before it can be used in practice for plant-derived metabolite production (Stöckigt et al., 1995).

Methyl jasmonate and sucrose stimulate in vitro phenolic production

The formation of secondary products in plant cell culture can be stimulated by biotic and abiotic elicitors. These elicitors act as stresses, and stimulate defense responses and increase biosynthesis of secondary products in cell culture (Weathers et al., 2010). Various elicitors include biological extracts and extreme culture conditions, such as microorganism extracts, UV radiation, and high salinity (Dörnenburg and Knorr, 1995).

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are signal compounds that elicit plant stress responses and enhance plant secondary metabolite accumulation by regulating key enzyme activities (Sudha and Ravishankar, 2002). Therefore, JA and MeJA have been widely applied as elicitors to improve secondary metabolite production in various plant cell cultures. For example, JA increased phenolic compounds in Hypericum perforatum L. cell suspension cultures (Gadzovska et al., 2007), and was used
to stimulate taxol accumulation in taxus cell cultures (Collin, 2001; Malik et al., 2011). Methyl jasmonate stimulated shikonin formation in cultured *Onosma paniculatum* cells (Ding et al., 2004), Bilobalide and Ginkgolides in *Ginkgo biloba* cell cultures (Kang et al., 2006), artemisinin in *Artemisia annua* L. (Wang et al., 2010), and peruvoside in *Thevetia peruviana* cell suspension cultures (Zabala et al., 2010; Kim et al., 2006). In addition, MeJA increased total phenolic content in various species including sweet basil (Kim et al., 2006B; Li et al., 2007), lettuce (Kim et al., 2007), radish (Kim et al., 2006A), and *Salvia miltiorrhiza* (Xiao et al., 2009).

Sucrose affects secondary metabolite production by inducing osmotic stress or by influencing the primary metabolite production and then secondary metabolites (Chakraborty and Chattopadhyay, 2008). High sucrose concentrations in plant cell culture are likely to inhibit tissue growth but enhance secondary metabolite production (Collin and Edwards, 1998; Mathur et al., 2010). High sucrose concentrations increased phenols, flavonoids, chlorogenic acid, and total hypericin content in root suspension cultures of *Hypericum perforatum* L. (Cui et al., 2010), and induced phenolic accumulation in leaf discs of *Vitis vinifera* L. (Pirie and Mullins, 1976).

To summarize, as a potential medicinal herb for burn treatments, burdock contains phenolic compounds that are likely important in its pharmacology. Plant cell culture can be an efficient method to produce phenolics from burdock, without the variation from environmental and genetic sources that are common in field-grown plants. However, the procedures for burdock callus culture and optimization of culture conditions for *in vitro*
phenolic production are unknown. In addition, the profiles of phenolic compounds accumulated in burdock callus are unclear. More information about burdock phenolic production and the identification of theses metabolites is needed, before the practical application of burdock phenolics for treating burns can be developed.

Research objectives

The objective of this project was to develop efficient techniques for burdock phenolic metabolite production, which could ultimately be used to develop a reliable burn treatment. This research involved the development of techniques for burdock callus culture, the optimization of culture conditions to enhance the *in vitro* phenolic production from burdock callus, and then the analysis of phenolic compounds from burdock callus. In Chapter 2, development of the procedures for burdock callus culture will be described. In Chapter 3, studies on the effects of MeJA and sucrose on phenolic formation in burdock callus culture will be reported. In these studies, methods of stimulating phenolic production in burdock callus culture were optimized, and medically interesting phenolic compounds in burdock were preliminarily identified. Chapter 4 will present a summary and conclusion from this research, along with suggestions for further study.
References


Chapter 2: Callus Culture of Burdock

Summary

Burdock (Arctium lappa L.) has been used as an herbal medicine for treating burn wounds. Phenolic compounds are likely to be responsible for the effectiveness of burdock in the healing of burns. Plant cell culture can be an efficient method for production of medically useful phenolics found in burdock leaves. Studies were conducted to develop procedures for production and maintenance of burdock callus culture. Burdock cotyledon or leaf sections were cultured on three media supplemented with different concentrations and combinations of plant growth regulators including BA, 2, 4-D and NAA. Based on callus growth and browning severity on these media, the suitable callus induction medium contained MS salts supplemented with B5 Vitamin, 3% sucrose, 1 mg/L BA, 2 mg/L 2, 4-D, and 0.2% Gelrite™. The suitable callus long-term maintenance medium consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1.5 mg/L NAA, 1 mg/L 2, 4-D and 0.2% Gelrite™. The procedures of burdock callus culture were determined. Sterilized burdock cotyledon or leaf sections were cultured at 25±1°C in the dark on callus induction medium. Well-developed callus was separated and subcultured every 4 wks. Three months later, nearly colorless and fast growing callus was transferred to
callus maintenance medium and subcultured on the same callus maintenance medium every 4 wks.

Introduction

Plant cell culture has become a promising method for producing plant-derived medicinal metabolites under controlled culture conditions for decades. Cell culture enables secondary metabolite production to be independent from source plants, thus avoiding the inconsistencies in biochemical accumulation in source plants due to the variation in environment and genetics. In addition, plant cell cultures diversify plant metabolites and often produce more bioactive compounds than are easily obtained from intact plants (Staba, 1977). Secondary products play important protective and defensive roles in plants (Briskin, 2000), and their production can be stimulated reliably in vitro by compounds and conditions that induce stress responses. Therefore, plant cell culture can be used to enhance in vitro secondary metabolite accumulation from cell cultures by modifying culture conditions (Ramachandra and Ravishankar, 2002). The factors influencing biochemical production in plant cell culture include genotype, developmental stage of explants, and culture environment such as culture media, temperature and light.

Plant growth regulators play an important role in regulating growth and development in plant tissue culture. Auxins and cytokinins are the two classes of growth regulators generally required and most commonly used in plant tissue culture. The most commonly
used synthetic auxins are 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 1-naphthaleneacetic acid (NAA), which influence cell growth expansion and organogenesis. Indole-3-butyric acid (IBA) is a natural auxin commonly used in plant tissue culture, which is broken down in media several days after application. The most commonly used synthetic cytokinins are kinetin (KT) and 6-benzylaminopurine or benzyladenine (BA), which interact with auxins and stimulate cell division. Thidiazuron (TDZ) is another growth regulator which has cytokinin activity for shoot induction (Gaspar et al., 1996). No studies have been conducted to evaluate different combinations of these growth regulators for inducing and maintaining callus from burdock.

Burdock is a large biennial herb which grows throughout much of the northern U.S. and Canada, and is common along roadsides, waste areas, and sometimes in pastures (Gross et al., 1980). It has been used as a folk medicine for treating burns in the Amish community in the U.S., where Amish healers apply burdock leaves as a bandage to burn wounds. Phenolics in burdock leaves are the potential medically useful compounds for burn treatment since phenolics have diverse medicinal applications including antioxidant, anti-inflammatory, and anti-bacterial activities (Shetty and McCue, 2003). To produce stable amounts and species of pheneolics from burdock for burn treatment, plant cell culture can be an efficient method as described above. In this project, burdock callus was induced and used as the materials for in vitro phenolic production. Production of burdock callus cultures has never been reported. For production of burdock callus culture, existing information on the culture of related species could be useful. For example, in sunflower (Helianthus annuus L.), which is in the same family as burdock, BA and NAA have been
used to induce callus (Greco et al., 1984; Espinasse et al., 1989). In *Onopordum turcicum*, callus was induced in the presence of 2, 4-D, NAA, BA and KT (Tamer and Mavituna, 1996). For this current research, preliminary studies were conducted to initially screen different combinations and concentrations of growth regulators for burdock callus culture.

The objective of this research was to develop procedures for burdock callus culture. To select the optimum media for burdock callus induction and long-term maintenance, burdock explants were cultured on media supplemented with different concentrations and combinations of plant growth regulators, and callus growth responses to these media were evaluated.

**Materials and Methods**

*Plant materials and culture conditions*

Burdock seeds were Watanabe Early variety and obtained from B & T World Seeds. Preliminary studies were conducted using various burdock explants to induce callus, including cotyledons and leaves cut into different sizes with or without mid-vein and margins, petiole segments and stem segments. The most suitable burdock explants for inducing callus culture were cotyledon and leaf sections of about 0.25 cm² without mid-
vein and margins. Conditions suitable for burdock callus induction and maintenance were on solid medium at 25±1°C in the dark.

Preliminary studies tested various media for burdock callus culture. All the media tested consisted of Murashige and Skoog (MS) salts (1962) supplemented with Gamborg’s B5 Vitamin (1968), 3% sucrose, 0.2% Gelrite™ and different concentrations and combinations of commonly used growth regulators, including one or two of the following: 1, 1.5, 2, 2.5, 4 mg/L BA, 1, 1.5, 2 mg/L KT, 1.5, 2 mg/L TDZ, 0.2, 0.5, 1, 1.5, 2.5 mg/L NAA, 0.5 mg/L IBA, and 0.2, 0.5, 1, 2, 4, 5, 8, 10 mg/L 2, 4-D. Media were adjusted to pH 5.7 and autoclaved for 20 min at 121°C at 1.1 kg/cm² pressure.

Three media, designated as M 1, M 2, and M 3 (Table 1), consisted of 1.5 mg/L BA and 1 mg/L NAA (M 1), 1 mg/L BA and 2 mg/L 2, 4-D (M 2), and 1.5 mg/L NAA and 1 mg/L 2, 4-D (M 3). Burdock leaf sections cultured on these three media had 100% callus formation frequency; therefore these three media were selected and used for the following studies on callus induction and maintenance to develop suitable procedures for callus culture based on callus growth and browning responses.
Callus induction

Burdock cotyledons from sterile seedlings were collected 4 days after cotyledon enlargement and used for callus induction. Burdock seeds were surface-sterilized in 30% CLOROX regular bleach (containing 6% sodium hypochlorite) on a shaker for 30 min, then rinsed in reversed osmosis/deionized (RO/DI) water 5 x in a laminar flow hood. Pairs of seeds were placed in a Magenta box with 50 ml medium consisting of MS salts supplemented with B5 Vitamin, 3% sucrose, and 0.2% Gelrite™, and incubated in a 16 h light/8 h dark photo period at 50 µmol m⁻² s⁻¹ at 25±1 C. Aseptically germinated seedlings were selected for tissue culture. The selected plants were those appearing vigorous and at similar growth stage. Cotyledons were excised, cut into similar pieces of 0.25 cm² in size without margins or mid-vein, and used for inoculation onto media.

Cotyledon sections were cultured on the three media described above (Table 1) at 25±1 C in the dark. A completely randomized experiment was conducted with the three media as treatments and three replications. Nine pieces of cotyledon sections were placed in each Petri dish and there were three Petri dishes for each medium, representing the three replicates with nine subsamples in each treatment. Callus development was assessed every wk over the first 5 wks by measuring the fresh weight of cotyledon sections initially at the time of inoculation (initial weight) and at the end of each wk after inoculation (weight at wk n) for 5 wks.
All data reported here were the means of three replications. Callus growth was expressed in the following two ways: fresh weight and callus relative growth. Callus relative growth at the end of each wk was calculated using the initial fresh weight at inoculation and fresh weight at wk n by the following formula:

\[
\text{Callus relative growth} = \frac{\text{fresh weight at wk } n - \text{initial fresh weight}}{\text{initial fresh weight}}
\]

Data for callus relative growth were tested for normality, and transformations were performed when needed to meet the assumptions of analysis of variance. Differences in callus relative growth among three media at each wk were tested by one-way ANOVA performed by Sigma Plot. Multiple comparisons for all pairs of means (Holm-Sidak method) were performed with an overall significance level of 0.05.

**Callus maintenance**

Burdock callus was maintained from the callus obtained from burdock leaves excised from two-mo old seedlings grown at The Ohio State University, Department of Horticulture and Crop Science greenhouse (Figure 1). The selected plants were those appearing vigorous and at similar growth stage, with no signs of disease or pest problems (Gamborg and Phillips, 1995). Freshly harvested leaves were disinfected with 70% ethanol for 20 s, followed by 5% Clorox regular bleach (containing 6% sodium hypochlorite) for 10 min, and rinsed with RO/DI water 5 x under aseptic conditions in a
laminar flow hood. Sterilized leaves were cut into 0.25 cm² pieces without leaf margins or mid-vein, and used for inoculation.

Leaf sections were cultured on the three media described above (Table 1) at 25±1°C in dark. Four wks after inoculation, well-developed callus was separated and transferred on the same media for long-term maintenance and subcultured every 4 wks. Since medium M 3 showed least browning, part of the callus induced on M 1 and M 2 was subcultured on M 3 at the 3rd subculture. Callus browning responses were evaluated visually at the 10th subculture.

Results

Callus induction

High callus induction frequency and fast, continual callus growth are the two criteria for selecting callus induction media. Callus induction frequency from burdock cotyledon sections was 100% on all the three media (M 1, M 2, and M 3), but the growth of callus varied with the media. Callus growth was quantified as the fresh weight of cotyledon sections over the first 5 wks after inoculation, which was measured wkly. On M 1, callus exhibited a sigmoidal growth pattern (Figure 2). Following a slow increase in fresh weight in the first wk after inoculation, callus quickly grew (0.08 g/wk) from the second
wk to the fourth wk, and then the increase in fresh weight slowed (0.06 g/wk) in the fifth wk. The burdock callus growth curve on medium M 1 could be divided into three growth phases, a lag phase (wk 1), exponential phase (wk 2-4), and early stationary phase (wk 5) (Liu and Moriguchi, 2007). On medium M 2, callus growth appeared to stay in the lag phase for the first 4 wks after inoculation (0.02 g/wk), but the growth rate in the fifth wk (0.07 g/wk) exceeded that on M 1 (0.06 g/wk). On medium M 3, callus growth stayed in the lag phase for the first 5 wks after inoculation, with growth rates of less than 0.01 g/wk. During the first 5 wks after inoculation, no serious browning response was observed on all of the three media.

Relative growth of callus induced on different media was different (p=0.05) at every wk of measurement (Figure 3). Callus on medium M 1 had the highest relative growth, followed by medium M 2, and medium M 3 showed the lowest callus relative growth, which suggests that among the three media tested, medium M 1 had the fastest callus development from burdock cotyledon sections over 5 wks after inoculation.

Callus maintenance

The goal of long-term callus maintenance is to culture stable and continually growing callus that can be stored and harvested over a period of time. Browning is a major problem in callus culture, which is due to the oxidation of polyphenols (He et al., 2009). Browning has toxic effects on callus cultures, reduces callus growth, and leads to the
death of callus (Zaid, 1987; Laukkanen et al., 2000); therefore, browning response is an important criterion for selecting a suitable medium for callus long-term maintenance.

When burdock callus induced from the three different media was used for long-term maintenance with subculture every 4 wks, browning responses were different. Callus induced on medium M 1 showed serious browning resulting in callus death (A in Figure 4), callus induced on medium M 2 showed less browning (C in Figure 4), and callus induced on medium M 3 had no browning (E in Figure 4). When callus induced on media M 1 and M 2 was transferred to medium M 3, M 1-induced callus remained fatal browning (B in Figure 4), but M 2-induced callus was maintained with slight browning which was not a problem for long-term maintenance (D in Figure 4). M 1-induced callus stopped growing, either maintained on M 1 or transferred to M 3. M 2 induced and maintained callus showed little growth, but M 2-induced callus continually grew after transferred to M 3. M 3 induced and maintained callus showed continual growth in the long term. Therefore, medium M 3 appeared to be the best for long-term callus maintenance. Callus induced from media M 2 and M 3 was able to be maintained on M 3 without a browning problem.
Discussion

In plant tissue culture, concerted and cooperative activities of plant hormones or growth regulators are usually required for tissue growth and development. Among the numerous plant natural hormones or synthetic growth regulators, auxins and cytokinins and their interactions are the most important for cell growth and division, thus both auxins and cytokinins are generally needed in plant tissue culture (Gaspar et al., 1996). In the preliminary studies and then the callus induction study, media tested were supplemented with combinations of one auxin from NAA, IBA or 2, 4-D and one cytokinin from BA, KT or TDZ, or either one auxin or one cytokinin. Results showed that combination of 1.5 mg/L BA and 1 mg/L NAA (M 1) exhibited fastest callus development, followed by combination of 1 mg/L BA and 2 mg/L 2, 4-D (M 2). Cytokinins KT and TDZ, and auxins IBA were not effective for burdock callus induction. These results were essentially consistent with other species in the same family, such as sunflower, which requires BA and NAA for callus induction (Greco et al., 1984; Espinasse et al., 1989), and Onopordum turchicum which needs 2, 4-D, NAA, BA and KT to induce callus (Tamer and Mavituna, 1996).

Burdock callus showed rapid and stable growing for long-term maintenance when they were nearly colorless without serious browning problems. When callus had browning problems, growth slowed, and eventually, callus died. Therefore, browning was a major problem for burdock callus culture in the long-term. Some studies have suggested that auxins inhibit browning whereas cytokinins stimulate phenolic synthesis and thus
enhance browning (Zaid, 1987). A medium containing 1 mg/L 2, 4-D and 0.2 mg/L NAA, similar to M 3 in this study, was found to be the most suitable for long-term culture of callus from Lycium barbarum L. (Ratushnyak et al., 1990). Consistent with these previous results, in the callus maintenance study, media M 1 and M 2 with cytokinins had browning problems, and medium M 3 supplemented with only auxins (2, 4-D and NAA) had no browning problems. Ascorbic acid was used to prevent or reduce browning in various species in tissue culture (Ko et al., 2009). To further reduce callus browning, filter sterilized ascorbic acid can be added to autoclaved media.

Although medium M 1 induced callus from burdock leaf sections at the highest growth rate, the callus induced on M 1 showed a serious browning problem and could not be used for long-term maintenance. Medium M 2 induced callus rapidly and the callus could be transferred to M 3 and used for long-term maintenance without serious browning issues. Therefore, medium M 2 could be used for callus induction. Medium M 3 exhibited low callus growth, but it was the best medium for long-term maintenance, so it could be used as callus maintenance medium for preparing stably growing and non-browning callus in the long-term.

Based on these studies, procedures of induction and maintenance of callus from burdock cotyledons and leaves were summarized as Figure 5. Sterilized burdock cotyledon or leaf sections 0.25 cm² in size were cultured at 25±1°C in dark on callus induction medium, which consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1 mg/L BA, 2 mg/L 2, 4-D, and 0.2% Gelrite™. All media were adjusted to pH 5.7 and autoclaved for
20 min at 121 C, 1.1 kg/cm². Well-developed callus was separated and subcultured every 4 wks. Three mos later, nearly colorless and fast growing callus was transferred to callus maintenance medium, which consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1.5 mg/L NAA, 1 mg/L 2, 4-D and 0.2% Gelrite™. Callus was subcultured on the same callus maintenance medium every 4 wks until used.
References


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<tr>
<th>Medium</th>
<th>BA</th>
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<tr>
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Table 1 Media used for studies on callus induction and maintenance.
Figure 1 The two-mo-old burdock seedling growing in the greenhouse used for callus maintenance study.
Figure 2 Growth curve of burdock callus on the three media (M 1, M 2, and M 3) based on fresh weight. Data represented average of three replications, bars indicate standard error.
Figure 3 Growth curve of burdock callus on the three media (M 1, M 2, and M 3) based on callus relative growth. Data represented average of three replications, bars indicate standard error.
Figure 4 Browning responses of burdock callus induced and maintained on different media. A: induced and maintained on M 1; B: induced on M 1, then maintained on M 3; C: induced and maintained on M 2; D: induced on M 2, then maintained on M 3; E: induced and maintained on M 3.
Figure 5 Procedures of induction and maintenance of callus cultures from burdock.

- Callus induction medium (M 2): 1 mg/L BA & 2 mg/L 2, 4-D
- Separated and subcultured every 4 weeks
- Callus maintenance medium (M 3): 1.5 mg/L NAA & 1 mg/L 2, 4-D
- Subcultured every 4 weeks until used
Chapter 3 Effects of Methyl Jasmonate and Sucrose on Phenolic Accumulation, and Phenolic Profiles in Burdock Callus Culture

Summary

Burdock (*Arctium lappa* L.) is a biennial plant used as a folk medicine by Amish people in the U.S. to treat burn wounds. The mechanism of burdock in healing and alleviating burns is unknown, as are the phytochemicals in burdock which are useful for burn treatment. This study was aimed at isolating and identifying phytochemicals from burdock that might possess medical functions in the burn healing process, and developing the technology of their production. We focused on phenolic compounds in burdock as our target metabolites based on the various biological properties of phenolics which are likely to be medically useful for burn treatment. We used burdock callus induced from leaves as the material to produce phenolics, and added different concentrations of MeJA and sucrose to the culture media to enhance phenolic production. We measured the total phenolic content by the Folin-Cuicaktey assay, and quantified and preliminarily identified the individual phenolic compounds from burdock callus using reversed-phase HPLC-DAD methods. We found that MeJA up to 1 mM and high concentrations of sucrose (60 and 90 g/L) increased phenolic accumulation, but decreased callus growth.
Considering both of the effects on phenolic content and callus biomass, 0.1 or 1 mM MeJA and 30 g/L sucrose were the optimum conditions for high phenolic yield in burdock callus. Based on the HPLC results of retention times and UV-spectra, the major phenolic compounds detected were preliminarily identified to be hydroxycinnamic acid derivatives.

Introduction

Burdock has been used as a folk medicine for treating burns in the Amish communities in the U.S for decades. The medically functional compounds in burdock for burns are likely to be phenolics because of their antioxidant and anti-inflammatory properties (Shetty and McCue, 2003; Shetty and Wahlqvist, 2004) and the high content of phenolic compounds in burdock (Lou et al., 2010B). No existing published research was dedicated to understanding the phenolic profiles in burdock associated with burn treatments and developing the methods for producing these compounds.

Total phenolics and individual phenolic compounds can be determined by spectrometry and chromatography methods. Total phenolic content is most commonly determined by the Folin-Ciocalteu assay (Xu and Chang, 2009; Henríquez et al., 2010; Milella, 2011). The Mo- and W-containing Folin-Ciocalteu (FC) reagent changes color from yellow to blue when it is reduced by phenolic compounds. The depth and intensity of blue color represents the ability of phenolics to reduce the FC reagent, which is proportional to the
concentration of phenolics in the extract. Therefore, total phenolic content can be determined by measuring the blue color absorbance through a spectrophotometer.

Individual phenolic compounds can be quantified and identified by High Performance Liquid Chromatography (HPLC). HPLC is one of the most widely used tools in analytical chemistry, and very popular in plant biochemical analysis (Allwood and Goodacre, 2010). Components of a mixture can be separated by HPLC based on their different affinities for the column stationary phase and liquid solvent mobile phase. Components more attracted to the stationary phase remain in the system longer and thus elute later than those that prefer the mobile phase, resulting in different eluting peaks. HPLC has been used to separate mixtures of compounds, identify individual compounds based on the time a compound elutes from the column (retention time), and quantify individual compounds based on the detected chromatographic peak areas. The HPLC system can be equipped with different detectors, and the photo diode array detector (DAD) is commonly used. Eluted sample components are detected as absorbance readings at a variety of wavelengths, resulting in spectra as adsorption against wavelength. The spectrum of a compound can be associated with its retention time to enhance the reliability of identification (Vrsaljko et al., 2012).

Plant cell culture can be used to produce phenolics from burdock in a reliable and efficient way. Using plant cell culture to produce plant secondary products in vitro is more controllable and stable compared to field-grown plants, which are subject to great environmental and genetic variation. In addition, plant cell culture can be more efficient
than field production for plant-derived metabolite production because the accumulation of secondary products can be significantly enhanced in plant cell culture by optimizing the *in vitro* culture conditions and adding various elicitors (Staba, 1997).

The formation of plant secondary products in cell culture can be stimulated by biotic and abiotic elicitors. Stresses can stimulate defense response and increase biosynthesis of secondary products in cell cultures. Methyl jasmonate is a signaling compound that elicits plant stress resistance response, and has been widely used to enhance secondary metabolites in plant cell cultures (Sudha and Ravishankar, 2002). Sucrose, as the carbon source in the medium, can influence secondary metabolites by inducing osmotic stress or by affecting the primary metabolite production. Therefore, supplementing appropriate concentrations of MeJA or modifying the concentrations of sucrose in media can result in high phenolic production from burdock callus culture.

In our previous studies, we developed the procedures for inducing and maintaining callus culture from burdock cotyledon or leaf sections (Chapter 2). Sterile burdock cotyledon or leaf sections were cultured at 25±1C in the dark on the medium consisting of MS salts supplemented with B5 Vitamin, 3% sucrose, 1 mg/L BA, 2 mg/L 2, 4-D, and 0.2% Gelrite™. Well-developed callus was separated and subcultured every 4 wks. Three mos later, nearly colorless and fast growing callus was transferred to a callus maintenance medium, which consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1.5 mg/L NAA, 1 mg/L 2, 4-D and 0.2% Gelrite™, with subculture every 4 wks. We also preliminarily analyzed the phenolic profiles in burdock callus by HPLC. Results showed
that burdock callus accumulated diverse phenolic compounds, which were consistent with those from field-grown plants. Burdock callus culture is likely to be a method for producing medicinal phenolics. However, burdock callus culture needs to achieve a commercially significant productivity to be used for practical production, and the developed method for burdock callus culture is not efficient enough. Before burdock callus culture can be applied to practical phenolic production for burn treatment, culture media must be modified for a higher phenolic productivity by adding elicitors such as MeJA and high concentrations of sucrose. In addition, qualitative and quantified analysis of the phenolic compounds accumulated in burdock callus is needed before these secondary products from burdock callus can be used in medicine.

The objectives of this study were to:

1. Evaluate the effects of MeJA and sucrose concentrations on phenolic accumulation in burdock callus culture;

2. Determine the appropriate concentrations of MeJA and sucrose in media for high phenolic production;

3. Analyze the phenolic profiles from burdock callus.

Burdock callus was cultured on media supplemented with different concentrations of MeJA and sucrose. Callus growth was assessed by measuring callus fresh weight wkly. Total phenolic content in callus was determined, and the individual phenolic compounds were identified and quantified by HPLC.
Materials and Methods

Reagent, materials and culture conditions

Methyl jasmonate (95%), filters and syringes were purchased from Sigma Aldrich. Standards of chlorogenic acid were obtained from MP Biomedicals. Ultra-pure water was prepared with Thermo Scientific Nanopure. All aqueous solutions for HPLC were prepared using HPLC-grade water and were obtained from Fisher Scientific. All solvents for HPLC were of quality HPLC-grade and were obtained from Fisher Scientific.

Uniform and continually growing burdock callus was prepared by the procedures described above. In the MeJA study and the sucrose study, burdock callus was cultured at 25±1 C in dark. The basic media used for all treatments consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1.5 mg/L BA, 1 mg/L NAA, and 0.2% Gelrite™, which resulted in the fastest callus growth and highest phenolic accumulation (Figure 6) in the short term among the three media have been studied according to our preliminary data (Chapter 2).
Ten-mo-old (after initial inoculation) callus (Figure 7) was used as the material for evaluating the effects of MeJA on phenolic production in burdock callus culture. Initiated callus was isolated into similar size (about 0.2 g a piece) and cultured on media of different treatments.

To determine the effects of MeJA on phenolic production in burdock callus, a MeJA study was designed as a completely randomized experiment with five MeJA levels and three replications. Media consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1.5 mg/L BA, 1 mg/L NAA, 0.2% Gelrite™ and five concentrations of MeJA: 0, 0.01, 0.1, 1, 10 mM. Nine pieces of isolated callus were placed on each Petri dish, and there were twelve Petri dishes for each MeJA level, representing twelve samples with nine subsamples for each treatment. Three samples of each treatment were harvested randomly at the end of each wk for 4 wks. Callus fresh weight and total phenolic content were measured, and phenolic profiles were analyzed as described below.
Effects of sucrose concentrations on phenolic production in burdock callus culture

Eleven-mo-old (after initial inoculation) callus was used to evaluate the effects of sucrose concentrations on phenolic production in burdock callus culture. Initiated callus was isolated into similar size (about 0.2 g a piece) and cultured on media of different treatments.

To determine the effects of sucrose concentrations on phenolic production in burdock callus, a sucrose study was designed as a completely randomized experiment with three sucrose levels and five replications. Media consisted of MS salts supplemented with B5 Vitamin, 1.5 mg/L BA, 1 mg/L NAA, 0.2% Gelrite™ and three concentrations of sucrose: 30, 60, 90 g/L. Nine pieces of isolated callus were placed on each Petri dish, and ten Petri dishes for each sucrose level, representing ten samples with nine subsamples for each treatment. Five samples, chosen at random, of each treatment were harvested 2 wks and 3 wks after treatment respectively. Callus fresh weight and total phenolic content were measured, and phenolic profiles were analyzed.

Methods for phenolic extraction

Total phenolics were extracted using a method modified from procedures of fruit phenolic extraction in Dr. Joseph C. Scheerens’ lab (Ozgen et al., 2008). Callus from
each Petri dish was frozen by liquid nitrogen, ground by mortar and pestle, and mixed. One gram callus was then weighed and collected into a 50 mL Falcon tube as one sample. For total phenolic extraction, 20 mL of acetone, water, and acetic acid solution (70:29.5:0.5 v/v) was added to each sample and agitated to a suspension on the shaker for 30 min. After centrifugation for 15 min at 7500 rpm, supernatant was captured in a 250 mL filtering flask via suction filtration through Whatman No. 1 filter paper. The pellet was re-extracted with 15 mL extraction solvent twice more. After final extraction, filtrate was transferred to a round-bottom boiling flask and concentrated using a rotary evaporator system under partial vacuum (via aspirator) at a water bath temperature of 38±1 C until acetone and acetic acid odors were absent. Then the solution was transferred to a 10 mL volumetric flask and brought to volume with water. The acetone extracts were divided into two portions. One portion (1 mL) was used for total phenolic content determination. Another portion (9 mL) was transferred to a separatory funnel, with the addition of 9 mL 0.2 M sodium acetate, and then 15 mL ethyl acetate. After careful agitation, the lower phase was removed and the upper phase was collected in a glass tube. The removed lower phase was re-extracted twice with 10 mL and then 5 mL ethyl acetate. The upper phase was collected in the same glass tube and dried by nitrogen at 38±1 C until dryness. The dried ethyl acetate extraction was used for HPLC analysis.
**Determination of total phenolic content**

Total phenolic content was measured spectrophotometrically by the Folin-Cuicaktey assay (Singleton et al, 1999). An aliquot of 0.25 mL of acetone extract prepared above was mixed with 0.25 mL of FC reagent and 5.75 mL double distilled (DD) water, and allowed to stand for 8 min. After 8 min, 2.5 mL of 7% sodium carbonate solution was added and then 5 mL of DD water. Solutions were allowed to incubate for 2 hours at room temperature before absorbance of the resulting blue color was measured at 750 nm against water as a blank. Measurements were compared to a standard curve of gallic acid ranging from 0-400 mg/L (r=0.99). The total phenolic content was expressed as mg gallic acid equivalent per g fresh weight of callus. Laboratory duplicates were performed for each acetone extract.

**Analysis of individual phenolic compounds by HPLC**

Individual phenolic compounds were analyzed by a reversed-phase HPLC System Gold 406A liquid chromatograph (Beckman Coulter, Inc., Fullerton, CA) equipped with diode array detector (model 168) and an autosampler (model 508) interfaced to an IBM computer with 32 Karat V.8.0 software (Beckman Coulter, Inc).
The dried ethyl acetate extract prepared as described above was re-dissolved in 0.5 mL 30% HPLC-grade CH$_3$CN and filtered by a 3 mL disposable luer-lock syringe attached with a disposable 0.45 μm nylon filter. Filtered extract was transferred to a 100 μL insert placed in a labeled brown chromatographic sample vial, and performed on a Phenomenex Gemini (C6-phenyl) column held at 30 C. The mobile phase consisted of 0.2% acetic acid in H$_2$O (solvent A) and 100% CH$_3$CN (solvent B). The initial injection volume was 50 μL. The flow rate was 0.7 mL/min. The solvent program was as follows: hold at 91% A and 9% B from 0 to 10 min; transition to 78% A and 22% B from 10 to 20 min; transition to 70% A and 30% B from 20 to 35 min; transition to 40% A and 60% B from 35 to 40 min; hold at 40% A and 60% B from 40 to 45 min; transition to 91% A and 9% B from 45 to 50 min; hold at 91% A and 9% B from 50 to 55 min. The detection wavelength was 320 nm.

Components were preliminarily identified by comparing their retention times and spectra with commercial standards. Quantification was performed by establishing a standard curve of chlorogenic acid. Individual phenolic content was expressed as mg chlorogenic acid equivalent per 100 g fresh weight of callus.

*Statistical analysis*

Callus relative growth was calculated as:
Callus relative growth (g/g) = (callus fresh weight at wk $n$ – initial callus fresh weight)/initial callus fresh weight

Total phenolic content was calculated as gallic acid equivalents by reference to the standard curve ($Y = 0.0016x – 0.017$, $R^2 = 0.999$), and expressed as mg gallic acid equivalent per g fresh weight of callus.

Total phenolic production was calculated as:

Total phenolic production (mg/g) = total phenolic content (mg/g FW) × callus relative growth (g/g).

Individual phenolic content was reported as chlorogenic acid equivalents by reference to the standard curve ($Y = 202.3827x + 0$, $R^2 = 1$), and expressed as mg chlorogenic acid equivalent per 100 g fresh weight of callus.

Data provided for callus relative growth, total phenolic content, total phenolic production, and individual phenolic content were the means of three replications in the MeJA study and five replications in the sucrose study, and analyzed by the GLIMMIX procedure in SAS 9.2 software. Multiple comparisons among treatments were conducted with the Least Squares Means test. Significance was assessed at alpha=0.05.
Results

Effects of MeJA on burdock callus growth and total phenolic content

Colorless and stably growing burdock callus was cultured on media supplemented with MeJA ranging from 0 to 10 mM. Except for the 10mM MeJA treatment, callus continued to grow in all treatments over 4 wks (callus relative growth in each wk was higher than the previous wk at p=0.05), but callus growth was inhibited in the presence of MeJA (Figure 8). Callus relative growth on the medium without MeJA (0mM) was higher than that on all the media with MeJA (p=0.05). Increasing concentrations of MeJA reduced the callus relative growth, except for 0.01 mM MeJA and 0.1 mM MeJA, which did not show significant difference (p=0.05). On media added with 10mM MeJA, callus growth was completely inhibited.

Total phenolic content was reported as mg gallic acid equivalent/g fresh weight of callus. Methyl jasmonate ranging from 0.01 mM to 1 mM enhanced total phenolic content in burdock callus (Figure 9). As the concentration of MeJA increased up to 1 mM, the total phenolic content increased. However, 10 mM MeJA reduced total phenolic content. Over the first 4 wks after treatment, total phenolic content increased within the first 2 wks, and decreased in the later 2 wks, meaning 2 wks after treatment was the best time to harvest callus for the highest total phenolic content. Considering both effects of MeJA concentration and time of harvest, callus cultured on media with 1 mM MeJA and
harvested 2 wks after treatment contained a higher total phenolic content than all the other treatments (p=0.05).

To summarize, MeJA played a contradictory role in total phenolic production in burdock callus culture. With the increase of MeJA concentrations in media, total phenolic content in burdock callus increased (except for 10 mM MeJA), but callus growth decreased.

*Effects of sucrose on burdock callus growth and total phenolic content*

As shown in Figure 10, burdock callus cultured on media added with increasing concentrations of sucrose from 30 to 90 g/L showed different growth (p=0.05). The treatment of 30g/L sucrose, which was the normal concentration of sucrose added to media, showed highest callus relative growth. Higher concentrations of sucrose inhibited callus growth. The higher concentration of sucrose, the more callus growth was inhibited. However, callus kept growing on all treatments (wk 3 showed a higher callus relative growth than wk 2 at p = 0.05).

In the sucrose study, total phenolic content (mg gallic acid equivalent per g fresh weight of callus) was higher 2 wks after treatment than 3 wks (Figure 11). No significant difference in total phenolic content was found among different sucrose concentrations 2 wks after treatments, whereas, 90 g/L and 60 g/L sucrose increased total phenolic content compared to 30 g/L sucrose 3 wks after treatments. When the interaction of sucrose
concentration and time after treatment was considered, only the treatment of 30 g/L sucrose in wk 3 showed lower total phenolic content than the other treatments, while all other treatments were not significantly different (p=0.05).

To summarize, higher concentrations of sucrose (60 and 90 g/L) increased total phenolic content in burdock callus 3 wks after treatments, but greatly inhibited callus growth.

*Optimization of MeJA and sucrose treatment for total phenolic production*

To increase total phenolic yield in callus culture, we attempted to enhance both phenolic accumulation and callus growth (Weathers et al., 2010). However, stress-inducing factors, MeJA and high concentrations of sucrose, did not fulfill both goals. Instead, MeJA and high concentrations of sucrose present in callus culture increased phenolic content but reduced callus growth. The concept of total phenolic production was introduced to evaluate both effects on phenolic content and callus growth.

The data of phenolic production in MeJA treatments are shown in Figure 12, taking the effects of MeJA on both phenolic content and callus growth into consideration. Burdock callus cultured on medium supplemented with 0.1 mM MeJA and harvested 3 wks after treatment, or cultured on medium with 1 mM MeJA and harvested 4 wks after treatments, produced highest amount of phenolics.
Total phenolic production was affected by sucrose concentration and harvest time after treatment (Figure 13). Burdock callus grown on 30 g/L sucrose and harvested either 2 or 3 wks after treatment produced the highest amount of total phenolics. With 30 g/L sucrose, total phenolic content was higher at the end of the second wk than that of the third wk (Figure 11); however, callus mass was higher at the end of the third wk than the second. Therefore, the total phenolic production in burdock callus 2 wks and 3 wks after treatment of 30 g/L sucrose turned out to be not significantly different.

*Individual phenolic profiles determined by HPLC*

Figure 14 shows the HPLC chromatogram of burdock callus extracts in MeJA and sucrose studies. The three peaks marked as A, B, C were the three major peaks that appeared in each sample. Compound A was much more concentrated than the others. The UV spectra of these three peaks seemed to be identical to that of the chlorogenic acid standard (Figure 15), but their retention times were different. Based on their similar UV-spectra and various retention times, we preliminarily identified these three compounds to be hydroxycinnamic acid derivatives. The other small peaks detected by HPLC in Figure 14 also had similar spectra to chlorogenic acid. These results suggest, tentatively, that diverse hydroxycinnamic acid derivatives accumulated in burdock callus.
Effects of MeJA and sucrose on individual phenolic content

The content of individual phenolic compounds was expressed as mg chlorogenic acid equivalent/100 g fresh weight of callus. The effects of MeJA on phenolic compound A content were similar to that on total phenolic content (Table 2). MeJA significantly increased compound A content, except for the 10 mM MeJA treatment. The higher concentration of MeJA up to 1 mM, the more compound A content increased. In all treatments except for 10 mM MeJA, the content of compound A increased in the first 2 wks after treatment, and then decreased in the later 2 wks. Burdock callus cultured on 1 mM MeJA and harvested 2 wks after treatment produced the highest content of compound A.

For compound B (Table 3), only 0.1 and 1 mM MeJA enhanced its content (1 mM MeJA was more effective than 0.1 mM). The content of compound B increased in the first 2 wks after treatment, and dramatically decreased in the third and fourth wk. The best treatment for the highest content of compound B was 1 mM MeJA and harvested at the end of the second wk.

For compound C (Table 4), the content increased with the increase of MeJA concentrations up to 1 mM, whereas 10 mM MeJA did not have a significant effect on compound C content compared to control. The content of compound C increased in the first 3 wks after treatment, and decreased in the fourth wk. The optimum condition for the
The highest compound C content was cultured with 1 mM MeJA and harvested 3 wks after treatment.

The effects of sucrose concentrations on the content of compounds A, B and C varied. For compound A accumulation (Table 5), there was no significant difference between sucrose concentrations of 30 g/L and 90 g/L 2 wks after treatment, whereas 60 g/L of sucrose showed a lower content of compound A. Three wks after treatment, higher concentrations of sucrose (60 g/L and 90 g/L) increased the content of compound A. The treatments had the highest compound A content were 90 g/L sucrose and harvested either 2 or 3 wks after treatment, or 30 g/L sucrose and harvested 2 wks after treatment.

For compound B (Table 6), there was no difference among different concentrations of sucrose 2 wks after treatment. Three wks after treatment, the content of compound B increased with the increasing of sucrose concentration. Treatment with 90 g/L sucrose and harvested either 2 or 3 wks after application produced the highest content of compound B.

Two wks after treatment, 30 g/L sucrose produced a higher content of compound C than the higher concentrations of sucrose (Table 7). No significant difference was shown among treatments after 3 wks. The highest content of compound C could be achieved after 2 wks culture with 30 g/L sucrose, or after 3 wks culture with 60 or 90 g/L sucrose.
Discussion

Methyl jasmonate is a signal compound effective in increasing various secondary metabolites in a wide variety of plants. The role of MeJA signaling involves inducing stress related genes, enhancing defense secondary products, and eliciting defense responses (Sudha and Ravishankar, 2002). Results in this study showed that MeJA increased total phenolic content in burdock callus. The most effective treatment achieved nearly 6 times higher total phenolic content than control 2 wks after 1 mM MeJA application. The concentration of MeJA for the highest total phenolic content in burdock callus (1 mM) was close to the 0.5 mM MeJA concentration applied to other species such as sweet basil (Kim et al., 2006) and lettuce (Kim et al., 2007). In all MeJA treatments, total phenolic content increased in the first 2 wks after application and then decreased in the later 2 wks. The trend might be due to the oxidation of phenolics causing browning, or the volatility of MeJA leading to low MeJA remaining in the culture media in the later 2 wks, or the decrease in sucrose in media resulting in a lack of carbon source for secondary metabolism.

High concentrations of sucrose applied to plant callus culture can act as osmotic stress and induce defense response by increasing production of secondary metabolites. Alternatively, high sucrose might enhance secondary metabolite accumulation by influencing the primary metabolite production since sucrose serves as the carbon source for callus nutrient supply (Chakraborty and Chattopadhyay, 2008). High concentrations of sucrose (60 and 90 g/L) did not increase total phenolic content 2 wks after treatment,
but increased total phenolic content after 3 wks. This time-dependent effect suggested that sucrose concentrations affected total phenolic accumulation possibly by influencing the primary metabolite production. In other words, 3 wks after sucrose treatments, sucrose content in culture media decreased to become a limiting factor for secondary product accumulation, then treatment with a higher initial sucrose application produced higher content of total phenolics.

Treating callus culture with elicitors such as MeJA and high concentrations of sucrose, which were applied in this study, generally results in higher secondary metabolite accumulation but slower callus growth (Sudha and Ravishankar, 2002; Mathur et al., 2010). The results of this study are consistent with results of previous research showing that media supplemented with MeJA and high concentrations of sucrose enhanced total phenolic content, but reduced callus growth. To balance this conflict and select the optimum conditions for producing the highest amount of phenolics, the concept of total phenolic production was introduced. In the MeJA study, media supplemented with 1 mM MeJA produced the highest total phenolic content 2 wks after treatment. However, MeJA slowed callus growth with increasing concentration, which reduced total phenolic production. Therefore, the best condition for total phenolic production was not the same as for total phenolic content. To produce the highest amount of phenolics, burdock callus should be cultured on media supplemented with 0.1 mM or 1 mM MeJA and harvested 3 or 4 wks after treatments respectively. Similarly, in the sucrose study, even though high concentrations of sucrose (60 and 90 g/L) increased total phenolic content, they also hugely inhibited callus growth. In this case, 30 g/L sucrose, which was the normal
sucrose concentration in media, was the best for total phenolic production from burdock callus.

The content of individual phenolic compounds was affected by MeJA similarly to the total phenolic content. Accumulation of the three major compounds (marked as A, B, and C) increased with increasing concentrations of MeJA up to 1 mM. The condition for producing highest content of compound A and B was 2 wks after 1 mM MeJA treatment, which increased 13- and 58-fold respectively, whereas for compound C high content were produced 3 wks after 1 mM MeJA treatment and could achieve a 32-fold increase over the control. Even though MeJA has been used widely to increase secondary metabolites in plants, MeJA can affect different metabolites differently, instead of increasing every individual compound. For example, Kim, et al. (2007) found that MeJA increased caffeic acid content but reduced chlorogenic acid content in lettuce. The results in this study that MeJA increased all the major individual phenolic compounds might indicate that these individual phenolic metabolites were similar compounds or different derivatives of one compound.

The effects of concentrations of sucrose on individual phenolic compound accumulation were more complicated and less effective than those of MeJA. High concentrations of sucrose increased the content of compound A and B only after 3 wks of application (4-fold and 9-fold increase respectively), whereas there were no effects of high concentrations of sucrose on compound A and B accumulation 2 wks after treatment. The content of compound C was highest at the lower sucrose concentration (30 g/L) 2 wks
after treatment, nearly doubled compared to the higher concentrations (60 and 90 g/L). The difference of sucrose effects on these three compounds might be caused by the difference of carbon demand for their biosynthesis.

The major three phenolic compounds detected by HPLC were preliminarily identified as hydroxycinnamic acid derivatives based on their retention times and UV-spectra. Even though our identification needs to be confirmed by further analysis by more reliable methods such as GC-MS and LC-MS, our results are consistent with previous studies, which detected and identified various hydroxycinnamic acids from burdock (Ferracane et al., 2010; Lou et al., 2010A, B; Chen et al., 2004; Lin and Harnly, 2008).

Hydroxycinnamic acids are widely distributed in plants, and are known to have beneficial effects on human health (Gallardo et al., 2006; Korkina, 2007; Shahidi and Chandrasekara, 2010). Hydrocinnamic acids possess antioxidant activities (Chen and Ho, 1997; Nardini et al., 1995) and act as free radical scavengers. They have inhibitory activities on the growth of various micro-organisms such as yeasts, bacteria, fungi, and molds (Stead, 1993; Harris et al., 2010). In addition, hydroxycinnamic acids can reduce keloid and hypertrophic scars (Phan et al., 2003), and have potential application to skin protection (Graf, 1992) and cutaneous wound healing (Phan et al., 2001). These biological properties of hydroxycinnamic acids are possibly associated with the usefulness of burdock for burn treatment. Burn healing is a process involving inflammation, proliferation, and tissue remodeling, during which oxidative stress is important in pathophysiological alterations. Toxic reactive oxygen species and reactive
nitrogen species impair the wound healing process, and antioxidant therapy is beneficial in burn injury healing (Liu et al., 2008). The mechanism of burdock in healing burns might be due to the strong antioxidant properties of hydroxycinnamic acids, which appear to be abundant in burdock leaves. Another possible mechanism of burdock effects on burn wounds might be the anti-inflammatory functions of its hydroxycinnamic acids, since persistent inflammation can cause tissue damage and impair wound healing activity (Upadhyay et al., 2009). Anti-inflammatory activities of hydroxycinnamic acids might also contribute to the pain relief that healers experienced with burdock treatment (Summer et al., 2007). In addition, the antimicrobial effects of hydroxycinnamic acids might be also responsible for burn treatment to inhibit infection, and explain the usefulness of burdock in burn healing.
References


Henríquez, C., et al. (2010). Determination of antioxidant capacity, total phenolic content and mineral composition of different fruit tissue of five apple cultivars grown in Chile. *Chilean journal of agricultural research, 70*(4), 523-536.


<table>
<thead>
<tr>
<th>MeJA (mM)</th>
<th>Wk after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>7.87±2.70 c</td>
</tr>
<tr>
<td>0.01</td>
<td>29.61±2.17 c</td>
</tr>
<tr>
<td>0.1</td>
<td>57.65±11.63 b</td>
</tr>
<tr>
<td>1</td>
<td>93.42±14.66 a</td>
</tr>
<tr>
<td>10</td>
<td>2.84±0.55 c</td>
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</table>

Table 2 Effects of MeJA on compound A content. The content was expressed as mg chlorogenic acid equivalent per 100 gram of fresh callus. Data represented average of three independent replications ± standard error. Means in the same column with different letters were significantly different according to the Least Squares Means test (p=0.05).
<table>
<thead>
<tr>
<th>MeJA (mM)</th>
<th>Wk after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 0.50±0.20 c</td>
</tr>
<tr>
<td>0.01</td>
<td>1 2.17±0.33 c</td>
</tr>
<tr>
<td>0.1</td>
<td>1 5.31±0.69 b</td>
</tr>
<tr>
<td>1</td>
<td>1 12.98±1.29 a</td>
</tr>
<tr>
<td>10</td>
<td>1 0.39±0.05 c</td>
</tr>
</tbody>
</table>

Table 3: Effects of MeJA on compound B content. The content was expressed as mg chlorogenic acid equivalent per 100 gram of fresh callus. Data represented average of three independent replications ± standard error. Means in the same column with different letters were significantly different according to the Least Squares Means test (p=0.05).
<table>
<thead>
<tr>
<th>MeJA (mM)</th>
<th>Wk after treatment</th>
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<tbody>
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<td></td>
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</tr>
<tr>
<td>0</td>
<td>0.71±0.25 cd</td>
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<tr>
<td>0.01</td>
<td>1.66±0.24 bc</td>
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<tr>
<td>0.1</td>
<td>2.48±0.43 b</td>
</tr>
<tr>
<td>1</td>
<td>3.77±0.39 a</td>
</tr>
<tr>
<td>10</td>
<td>0.12±0.05 d</td>
</tr>
</tbody>
</table>

Table 4 Effects of MeJA on compound C content. The content was expressed as mg chlorogenic acid equivalent per 100 gram of fresh callus. Data represented average of three independent replications ± standard error. Means in the same column with different letters were significantly different according to the Least Squares Means test (p=0.05).
<table>
<thead>
<tr>
<th>sucrose (g/L)</th>
<th>wk after treatment</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>73.65±8.57 a</td>
<td>18.44±3.58 b</td>
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</tr>
<tr>
<td>60</td>
<td>37.90±8.27 b</td>
<td>46.37±10.61 ab</td>
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</tr>
<tr>
<td>90</td>
<td>58.81±12.33 ab</td>
<td>79.93±17.18 a</td>
<td></td>
</tr>
</tbody>
</table>

Table 5 Effects of sucrose concentrations on compound A content. The content was expressed as mg chlorogenic acid equivalent per 100 gram of fresh callus. Data represented average of five independent replications ± standard error. Means in the same column with different letters were significantly different according to the Least Squares Means test (p=0.05).
<table>
<thead>
<tr>
<th>sucrose (g/L)</th>
<th>wk after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>13.31±2.43 a</td>
</tr>
<tr>
<td>60</td>
<td>11.92±2.23 a</td>
</tr>
<tr>
<td>90</td>
<td>15.99±1.81 a</td>
</tr>
</tbody>
</table>

Table 6 Effects of sucrose concentrations on compound B content. The content was expressed as mg chlorogenic acid equivalent per 100 gram of fresh callus. Data represented average of five independent replications ± standard error. Means in the same column with different letters were significantly different according to the Least Squares Means test (p=0.05).
Table 7 Effects of sucrose concentrations on compound C content. The content was expressed as mg chlorogenic acid equivalent per 100 gram of fresh callus. Data represented average of five independent replications ± standard error. Means in the same column with different letters were significantly different according to the Least Squares Means test (p=0.05).

<table>
<thead>
<tr>
<th>Sucrose (g/L)</th>
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<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3.71±0.46 a</td>
<td>1.90±0.58 a</td>
</tr>
<tr>
<td>60</td>
<td>1.87±0.34 b</td>
<td>3.23±0.51 a</td>
</tr>
<tr>
<td>90</td>
<td>1.98±0.26 b</td>
<td>2.51±0.29 a</td>
</tr>
</tbody>
</table>
Figure 6 HPLC chromatogram of burdock callus extracts cultured on different media.
Figure 6 continued

- M 2

- M 3
Figure 7 Ten-mo-old burdock callus used for MeJA study.
Figure 8 Burdock callus relative growth 1 to 4 wks after being treated with different concentrations of MeJA. Data represented average of three replications, bars indicated standard error.
Figure 9 Total phenolic content in burdock callus 1 to 4 wks after being treated with different concentrations of MeJA. Data represented average of three replications, bars indicated standard error.
Figure 10 Burdock callus relative growth 2 and 3 wks after treatment with different concentrations of sucrose. Means in each wk with different letters were significantly different according to the Least Squares Means test (p=0.05). Data represented average of five replications, bars indicated standard error.
Figure 11 Total phenolic content in burdock callus 2 and 3 wks after treatment with different concentrations of sucrose. Means in each wk with different letters were significantly different according to the Least Squares Means test (p=0.05). Data represented average of five replications, bars indicated standard error.
Figure 12 Total phenolic production in burdock callus 1 to 4 wks after being treated with different concentrations of MeJA. Total phenolic production = phenolic content (mg/g FW) × callus relative growth (g/g). The treatment at 10 mM MeJA concentration had negative total phenolic production because callus relative growth was negative in this treatment. Data represented average of three replications, bars indicated standard error.
Figure 13 Total phenolic production in burdock callus 2 and 3 wks after being treated with different concentrations of sucrose. Total phenolic production = phenolic content (mg/g FW) × callus relative growth (g/g). Means in each wk with different letters were significantly different according to the Least Squares Means test (p=0.05). Data represented average of five replications, bars indicated standard error.
Figure 14 HPLC chromatogram of burdock callus extracts in MeJA and sucrose studies at 320 nm. Marked peaks A, B, C were the three major compounds of interest.
Figure 15 UV-spectra of chlorogenic acid standard and compound A, B, C.
Figure 15 continued

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**Compound B**

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**Compound C**

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Chapter 4 Summary, Conclusions and Recommendations

Burdock is a biennial plant, which has been used as a traditional medicine in China, and a health vegetable in Japan. In the U.S., burdock is generally regarded as a weed, but it has been used for burn treatment in the Amish communities. Amish people use burdock leaves as a bandage to cover burn wounds after salve application. This treatment has been reported to speed healing process, relieve pain when dressing is changed, and prevent bacterial infections. Even though this treatment is thought to be effective, it is not recognized as a standard treatment for burns, and efforts to understand its mechanism in burn healing and develop its practical application are lacking. In this study, we were aimed to understand the secondary metabolites in burdock which might be responsible for burn healing, and to develop techniques for producing these compounds for medicinal usage.

We used burdock callus as the material for medicinal metabolite production, and focused on phenolic compounds. The reason to use callus culture was that it is more controllable, stable, and efficient for plant-derived metabolite production compared with intact plants growing in the field. We focused on phenolics as the target compounds for two reasons: 1) phenolics possess medicinal functions such as antioxidant and anti-inflammatory activities, which are involved in the burn healing process; and 2) burdock contains high
amounts of phenolics. Both reasons indicate that phenolics in burdock are likely to be the compounds responsible for the effectiveness of burdock for treating burns. Therefore, we produced phenolic compounds in burdock callus culture, and analyzed the profiles of these phenolics.

We first developed the procedures for inducing and maintaining callus culture from burdock. We evaluated different explants, including burdock cotyledons, leaves, petioles, and stems, cut into different sizes, cultured in dark or in light, on media supplemented with different concentrations and combinations of growth regulators including BA, KT, TDZ, NAA, IBA, and 2, 4-D. A suitable procedure for burdock callus culture was developed, which showed high callus induction frequency and maintained fast and continually growing callus.

After the method for burdock callus culture was developed, we modified the culture media by adding different concentrations of MeJA and sucrose, aiming at enhancing phenolic productivity. We then analyzed phenolic compounds in burdock callus by determining total phenolic content through the Folin-Cuicaktey assay, and attempted to identify and quantify individual phenolic compounds by HPLC.

We successfully developed a suitable procedure for burdock callus culture. Burdock cotyledon or leaf sections were cut into 0.25 cm² in size and used as explants. Culture conditions were in dark at 25±1 C on medium consisted of MS salts supplemented with B5 Vitamin, 3% sucrose, 1 mg/L BA, 2 mg/L 2, 4-D, and 0.2% Gelrite™, with subculture every 4 wks. Three mos after initial inoculation, well-developed callus was
transferred to a new medium with different growth regulators, 1.5 mg/L NAA and 1 mg/L 2, 4-D. The callus was maintained on this medium and subcultured every 4 wks.

When MeJA and high concentrations of sucrose were added to culture media, callus growth was inhibited with the increase of concentrations of MeJA and sucrose. However, MeJA up to 1 mM and high concentrations of sucrose (60 and 90 g/L) increased both total phenolic content and major individual phenolic content. Optimum concentrations of MeJA and sucrose for total phenolic production were determined by considering both the effects on callus growth and total phenolic content, and calculating the product of callus relative growth and total phenolic content.

Phenolic compounds accumulated in burdock callus were preliminarily identified by HPLC as hydroxycinnamic acid derivatives based on their spectra compared with commercial standards. All of the three major peaks detected from HPLC had similar spectra but different retention times, which suggested that they are likely to be different hydroxycinnamic acid derivatives.

The conclusions we could draw based on our results: burdock callus can be used as the material for phenolic production; phenolic compounds accumulated in burdock callus were preliminarily designated to be hydroxycinnamic acid derivatives; appropriate concentrations of MeJA (0.1mM or 1 mM) added to culture media can increase total phenolic productivity; a commonly used concentration of sucrose (30 g/L) was the optimum concentration for high total phenolic production in burdock callus. Our study provided some information about the potentially medical useful compounds in burdock,
and developed a new and efficient method for their production. Our finding in this study laid a foundation for future studies on burdock metabolites that could be used to treat burns. The callus culture system we developed can be used as the research method for future studies, and it has the potential to be further improved for commercial production of medicinal phenolics from burdock.

We recognize two major limitations in our study. We decided to focus on phenolics as our target compounds to produce and analyze from burdock, based on the hypothesis that phenolics were the metabolites having medically functions for burn treatments. However, we did not know for sure what metabolites in burdock were actually responsible for its medical effectiveness. Assistance from the medical community will be necessary to solve this problem, which is beyond the scope of the studies described here. Another limitation was the semi-identification of individual phenolic compounds. With only the HPLC results, we could not identify the compounds with certainty. HPLC was not reliable enough since some compounds share very similar retention times and spectra.

Further studies are needed before burdock can be practically applied to burn treatments. Clinical studies are essential to have a better knowledge of the mechanism of burdock using for treating burns, and determine the specific metabolites in burdock that are associated with the medically effectiveness of burdock for burns. In addition, the compounds in burdock callus need to be identified using more reliable methods such as liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). These studies are ongoing in this laboratory. After the
identification of metabolites in burdock callus, they should be further compared with those metabolites accumulated in burdock plants grown in the field to verify that burdock callus culture could be used for medically useful metabolite production. Since our studies focused on phenolic acids, and medical functions of herbal medicine are usually contributed to the interactions of diverse classes of metabolites, it is necessary to have studies on the other classes of compounds in burdock to better explore its potential medicinal properties for burns. Our developed system for burdock callus culture can likely be improved, and possibly transferred to cell suspension culture systems, to meet commercialization requirements.


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