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ORIGINAL PAPER

# Effects of 2,4-dichlorophenoxyacetic acid combined to 6-Benzylaminopurine on callus induction, total phenolic and ascorbic acid production, and antioxidant activities in leaf tissue cultures of *Crataegus azarolus* L. var. *aronia*

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**Abstract** The present research work describes the effects of 2,4-dichlorophenoxyacetic acid (2,4-D)/Benzylaminopurine (BAP) ratio on callus induction, total phenolic and ascorbic acid production, and antioxidant activities in leaf-derived calli of *Crataegus azarolus* (hawthorn). The supplementation of 1.0 mg/L 2,4-D and 1.0 mg/L BAP to MS medium was found to be the most efficient for callus induction (as percentage and fresh weigh). The results of biochemical analysis showed that the highest total phenolic contents were obtained in callus cultured on MS medium supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BAP (52 ± 0.56 mg GAE/g DM) and were significantly lower than those of intact leaves (76 ± 1.72 mg GAE/g DM). However, the highest ascorbic acid contents were found in callus cultured on MS medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L BAP (0.96 ± 0.13 mg AAE/g DM) and these amounts were statistically similar to those found in leaf tissues (0.74 ± 0.07 mg AAE/g DM). Antioxidant activities of callus extracts were determined using two TEAC assays and results showed that extract of callus cultured on MS medium supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BAP have the greatest antiradical activities against DPPH (124 ± 2.92 mg TE/g DM) and ABTS (0.19 ± 0.02 mg TE/g DM) compared to the leaves of field-grown plant. Thus, the use of high level of 2,4-D over BAP can be suitable to enhance the quality more than the quantity of bioactive compounds in leaf callus culture of hawthorn.

**Keywords** Callus induction · Leaf-derived calli · Hawthorn · Chemical analysis · Antiradical activities

**Abbreviations**

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| AAE | Ascorbic acid equivalent |
| ABTS | 2,2′-Azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid |
| BAP | 6-Benzylaminopurine |
| DCIP | 2,6-Dichloroindophenol |
| DM | Dry matter |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| FW | Fresh weight |
| GAE | Gallic acid equivalent |
| IAA | Indole-3-acetic acid |
| KIN | Kinetin |
| MS | Murashige and Skoog |
| NAA | 1-Naphthaleneacetic acid |
| PAL | Phenylalanine ammonia-lyase |
| TAL | Tyrosine ammonia-lyase |
| TEAC | Trolox equivalent antioxidant capacity |
| TE | Trolox equivalent |

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## Introduction

The important commercial development of bioactive molecules has awarded a great interest to secondary metabolites due to their important antioxidant activities and the diversity of their biological properties. Their production in spontaneous plants is very limited by numerous problems such as the fluctuations of environmental factors and the abiotic constraints, difficult cultivation, deficient abundance (Matkowski 2008). Actually, the plant cell and tissue culture become an alternative potential means for the production and the accumulation of many valuable secondary metabolites because it can provide several benefits to minimize these problems allowing the continuous, reliable, and predictable production of these molecules independently of seasonal variations as well as the optimization and the enhancement of their biosynthesis under well controlled conditions (Simoés et al. 2009).

Many biotechnological approaches and various strategies of in vitro culture were used to enhance and stimulate the production of several bioactive compounds of medicinal plants (Ramachandra Rao and Ravishankar 2002). Most of them used the variation of combinations and concentrations of exogenous plant growth regulators especially auxins and cytokinins to modify and improve the accumulation of bioactive molecules (Duangporn and Siripong 2009). The ratio and type of growth regulators used are very crucial factors for the stimulation of biosynthetic pathways (Matkowski 2008). For example, the use of high concentration of 6-Benzylaminopurine (BAP, 1.0 mg/L) over 1-Naphthaleneacetic acid (0.1 mg/L) for the stem node callus culture of *Ecballium elaterium* was found to enhance the production of cucurbitacin B compared with original plant material (Toker et al. 2003).

The aromatic and medicinal plant chosen for this work is *Crataegus azarolus* (hawthorn), member of Rosaceae family. On the one hand, most species of hawthorn are characterized by a high potential chemical composition essentially including vitamin C, glycosides, saponins, tannins (Lakshmi et al. 2012), flavonoids, procyanidins (Bignami et al. 2003), anthocyanidins, and proanthocyanidins. These compounds have not only been used for their pharmacological and medicinal properties to treat many cardiovascular diseases (Verma et al. 2007) but also for their anti-nociceptive and anti-inflammatory effects (Bor et al. 2012). On the other hand, there are numerous studies of in vitro culture that has been conducted on genus of *Crataegus* showing good potentialities on callus tissue cultures that are able to produce various phenolic compounds (Bahorun et al. 2003; Bahri-Sahloul et al. 2014; Maharik et al. 2009).

In spite of these numerous researches, there is no available study about the influence of using plant growth regulators especially 2,4-dichlorophenoxyacetic acid (2,4-D) and BAP on antioxidant activities and production of total phenols and ascorbic acid of hawthorn callus cultures. The present study was carried out to optimize an efficient in vitro protocol for callus formation, in order to enhance the production of total phenols and ascorbic acid as well as the antioxidant activities from callus cultures of *C. azarolus* using different concentrations of auxin and cytokinin.

## Materials and methods

### Plant material

In April 2013, leafy twigs of adult hawthorn were harvested during the flowering stage from the region of Ain Drahem (Tunisian village characterized by humid mild winter climate and geographically situated at 800 m of altitude, 8° 41′ 05″ East of longitude, 36° 46′ 34″ North of latitude).

A first part of leaves was air-dried in the shade, and then crushed to obtain fine powder that was well-preserved in plastic hemolysis tubes away from heat and direct light, to be used as a control during the eventual future chemical analysis. Under laminar airflow cabinet, the second part of leaves was first washed with detergent and rinsed thoroughly with distilled water, then surface-sterilized using 70 % (v/v) of ethanol for 1 min, 5 mg/L of mercury chloride ($HgCl_2$) for 5 min, and 12 % of commercial solution of sodium hypochlorite for 10 min, and finally rinsed three times with sterile distilled water to be used as explants for the different callus cultures.

### Establishment of callus culture

After disinfection, small explants (3 × 2 mm) were excised from leaves and aseptically inoculated on MS medium (Murashige and Skoog 1962) solidified by 0.8 % of agar–agar, containing 3 % of sucrose and supplemented with 0.5, 1.0, and 2.0 mg/L of 2,4-D in combinations with 0.5 and 1.0 mg/L of BAP. The pH of medium was adjusted to 5.8 using sodium hydroxide (NaOH, 0.1 N) or hydrochloric acid (HCl, 1 N) before autoclaving at 112 °C for 30 min. Each explant was cultivated in test tube (20 mm in diameter and 150 mm in height) containing 20 ml of inclined medium and covered with steel lids and plastic film. Each culture included 24 explants (3 replicates × 8 tubes). All cultures were transferred into new fresh medium every 5 weeks and incubated in growth chamber, in darkness, at 26 ± 2 °C. After 15 weeks, callus samples were

collected from each culture, air-dried in the shade, and then stored for eventual future chemical analysis.

### Extraction

Half (1/2) gram of each dried sample (leaves or calli) was crushed with quartz using a mortar to obtain fine powder that was mixed with 5.0 ml of extraction solvent composed of acetone/distilled water/acetic acid (70:28:2, v/v/v). The mixture was shaken during 60 min at 4 °C and centrifuged a first time at 13,000 rpm for 15 min. The supernatant was removed and the residue of each sample was extracted and centrifuged a second time using the same protocol (Tabart et al. 2006). These two fractions of supernatant were combined together into Eppendorf tubes and stocked at −20 °C for chemical analysis.

### Determination of total phenolic contents

Determination of total phenolic contents was carried out according to the Folin−Ciocalteu reagent method described by Caboni et al. (1997). After appropriate dilution in distillated water, 30 µl of each extract was added to 3.6 ml of distilled water, and then mixed with 0.2 ml Folin–Ciocalteu reagent. Three minutes later, 0.8 ml of sodium carbonate (20 % w/v) was supplemented and the mixture was finally incubated at 100 °C for only one minute. After cooling, the absorbance was measured at 750 nm and the total phenolic contents were determined with linear calibration curve performed with gallic acid (25–500 mg/L) as standard. Results obtained were expressed in mg equivalent gallic acid (GAE) per gram of dry matter (DM). This dosage was performed in triplicate for each sample.

### Determination of ascorbic acid content

Determination of reduced ascorbic acid contents was effectuated according to the 2,6-dichloroindophenol (DCIP) method described by the Association of Vitamin Chemists (1961). A linear calibration curve was prepared with different concentrations of ascorbic acid (2.5–20 µg/mL) as standard. After an appropriate dilution with 5 % metaphosphoric acid, 0.6 ml of each extract was mixed with 0.5 ml of 10 % metaphosphoric acid, and then 0.3 ml citrate buffer (pH 4.15) and 0.3 ml DCIP (0.1 mg/mL) were added to the mixture. For each sample, optical density blanching was used and blank value was determined after the addition of 60 µL ascorbic acid (1 mg/mL) at 520 nm, in order to measure the interference due to sample color. Results obtained were expressed in mg equivalent ascorbic acid (AAE) per gram of dry matter (DM). This dosage was performed in triplicate for each sample.

### Evaluation of antioxidant activities

The first method used to examine the antioxidant capacity was the scavenging of the radical 2,2-diphenyl-1-picryhydrazyl (DPPH) as described by Tadolini et al. (2000). The stock solution of DPPH (75 mg/L) was prepared by stirring 15 mg DPPH in 200 mL methanol for 2 h in the dark. In this assay, 0.75 ml of diluted extract, standard (0–0.1 mM Trolox), or blank (methanol) was mixed with 1.5 ml DPPH solution. The absorbance was determined at 517 nm after 5 min incubation. For each sample, a blank with 1.5 ml methanol, instead of the DPPH reagent, was included to correct for any sample absorbance at 517 nm.

The second method used to evaluate the antioxidant capacity was the scavenging of the radical 2,2-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid, (ABTS) described by Re et al. (1999). The stock solution was prepared by stirring ABTS (7 mM) and potassium persulfate (2.45 mM) in water and allowing the mixture to stand in the dark for 12 to 16 h. Before use, this solution was diluted in ethanol to have an absorbance of 0.7 ± 0.020 at 734 nm, and then 10 µL of diluted extract, standard (0–0.1 mM Trolox), or blank (ethanol) were added to 1.0 ml ABTS solution. The absorbance was determined at 734 nm after 4 min. For each sample, a blank with 1.0 ml ethanol, instead of the ABTS reagent, was included to correct for any sample absorbance at 734 nm.

For these two assays, the antioxidant capacity was expressed in mg Trolox equivalent per gram of dry matter (mg TE/g DM). Each sample was analyzed in triplicate.

### Statistical analysis

Data of antioxidant capacity assays, as well as total phenolic and ascorbic acid contents were treated with the statistical analysis of the variance (ANOVA) to evaluate the significant differences between various callus samples of hawthorn. The difference was regarded as significant when $P \leq 0.05$.

## Results

### Effect of 2,4-D/BAP ratio on callus production

Leaf-derived calli of hawthorn grown on MS media supplemented with different concentrations of 2,4-D combined with different concentrations of BAP began to appear after 2–3 weeks. Data presented in Table 1 demonstrate the effects of using four different growth regulators combinations on percentage of calli formed and their fresh biomass. Results showed that MD2, MD3, and MD4 produced the maximum percentages of callus that were not significantly

**Table 1** Percentage and fresh weight of leaf-derived calli cultured on MS media supplemented with different concentrations of 2,4-D and BAP

| Media code | 2,4-D concentration (mg/L) | BAP concentration (mg/L) | Callusing percentage (%, mean ± SD) | Fresh weight (g, mean ± SD) |
|---|---|---|---|---|
| MD1 | 0.5 | 0.5 | $8.33 \pm 4.17^{b}$ | $0.07 \pm 0.03^{d}$ |
| MD2 | 1.0 | 0.5 | $95.83 \pm 4.17^{a}$ | $5.57 \pm 0.30^{b}$ |
| MD3 | 1.0 | 1.0 | $91.66 \pm 4.17^{a}$ | $6.93 \pm 0.29^{a}$ |
| MD4 | 2.0 | 1.0 | $95.83 \pm 4.17^{a}$ | $3.78 \pm 0.07^{c}$ |

Values represent mean ± SD of three repeated experiments. Data with different letters were significantly different ($P \leq 0.05$)

($P > 0.05$) different (95.8, 91.7, and 95.8 %); whereas the lowest value of callus formation was observed on MD1 (8.3 %). Furthermore, the highest value of callus production as fresh weight was attributed to MS medium supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L BAP (6.93 ± 0.29 g FW) compared with the other combinations. While the minimum callus fresh weight was recorded with MS medium containing 0.5 mg/L 2,4-D and 0.5 mg/L BAP (0.07 ± 0.03 g FW). Calli obtained from the different culture media are observed through Fig. 1a–d. Regardless of medium used, all calli observed were compact as texture and dark yellow colored. The biomass of calli grown on MD1 was insufficient to be used during the eventual future chemical analysis.

Effect of 2,4-D/BAP ratio on total phenolic contents

The effect of using different concentrations of 2,4-D and BAP in culture medium on the total phenolic contents in leaves of hawthorn (control) as well as their derived calli is shown in Fig. 2. Data revealed that on all media, callus contained total phenolic contents significantly lower than control. MS medium supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BAP (MD4) seemed to be the most suitable medium for the highest production of total phenols (52 ± 0.56 mg GAE/g DM) which was 32 % lower than control (76 ± 1.72 mg GAE/g DM). However, leaf-derived calli cultured on MS medium containing 1.0 mg/L 2,4-D and 1.0 mg/L BAP (MD3) gave the lowest value of phenolic contents (11 ± 1.96 mg GAE/g DM). These calli produced total phenols 7 times less than control.

Effect of 2,4-D/BAP ratio on ascorbic acid contents

The results of analysis of ascorbic acid in leaves of hawthorn and their derived calli grown on different culture media are illustrated in Fig. 3. As shown, the ascorbic acid contents were found to vary from 0.23 to 0.96 mg AAE/g

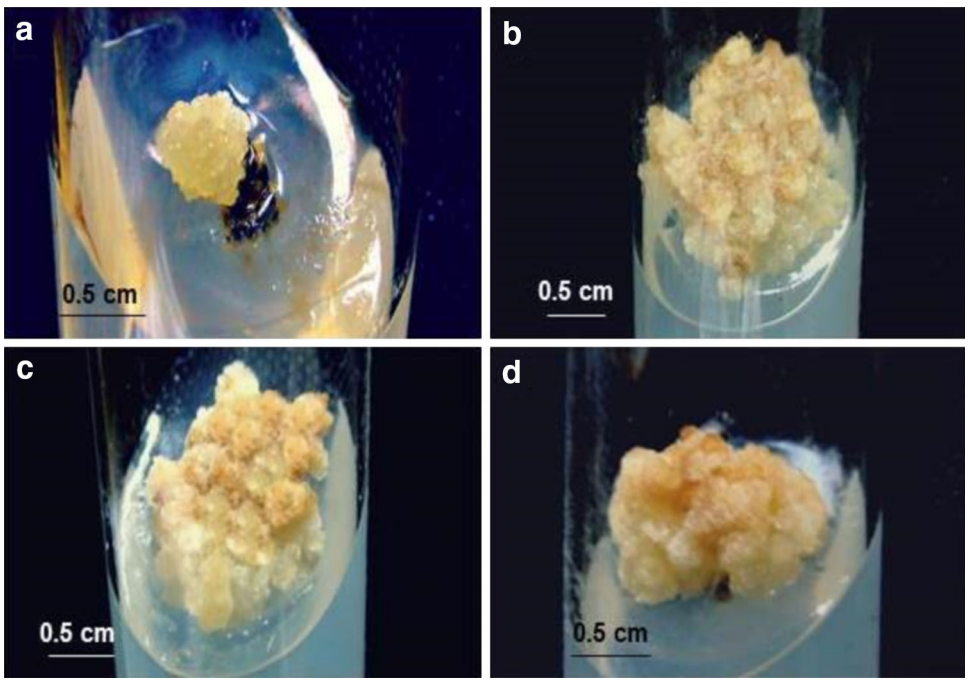


**Fig. 1** Callus grown on MS + 0.5 mg/L 2,4-D + 0.5 mg/L BAP (**a**), MS + 1.0 mg/L 2,4-D + 0.5 mg/L BAP (**b**), MS + 1.0 mg/L 2,4-D + 1 mg/L BAP (**c**), and MS + 2.0 mg/L 2,4-D + 1.0 mg/L BAP (**d**)

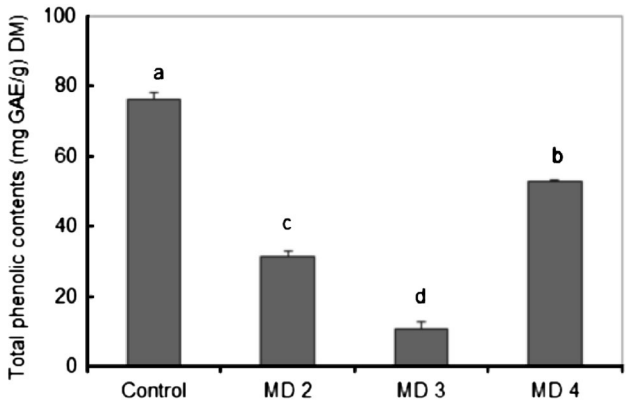


**Fig. 2** Total phenolic contents of hawthorn's leaves (control) and their derived calli grown on MS media supplemented with different concentrations of 2,4-D and BAP. Means with different letters were significantly different ($P \leq 0.05$). Values are mean ± SEM of three experiments. *MD1* MS + 0.5 mg/L 2,4-D + 0.5 mg/L BAP, *MD2* MS + 1.0 mg/L 2,4-D + 0.5 mg/L BAP, *MD3* MS + 1.0 mg/L 2,4-D + 1 mg/L BAP, *MD4* MS + 2.0 mg/L 2,4-D + 1.0 mg/L BAP

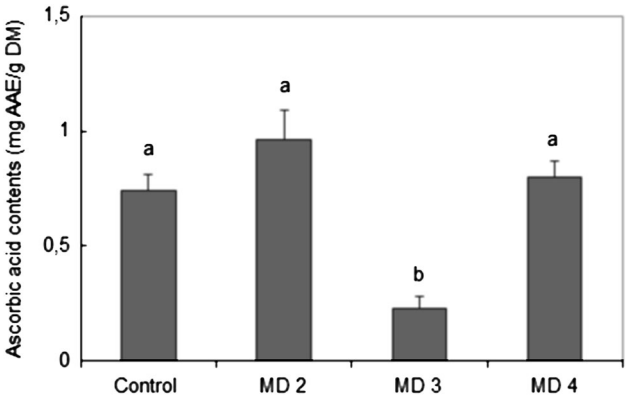


**Fig. 3** Ascorbic acid contents of hawthorn's leaves (control) and their derived calli grown on MS media supplemented with different concentrations of 2,4-D and BAP. Means with same letters were not significantly different ($P > 0.05$). Values are mean ± SEM of three experiments. *MD1* MS + 0.5 mg/L 2,4-D + 0.5 mg/L BAP, *MD2* MS + 1.0 mg/L 2,4-D + 0.5 mg/L BAP, *MD3* MS + 1.0 mg/L 2,4-D + 1 mg/L BAP, *MD4* MS + 2.0 mg/L 2,4-D + 1.0 mg/L BAP

DM according to the medium used. Similar values to control were found in callus cultured in the presence of 1.0 mg/L 2,4-D combined with 0.5 mg/L BAP (MD2) and 2.0 mg/L 2,4-D combined with 1.0 mg/L BAP (MD4); whereas the low values (3 times lower than control) were recorded with MS medium supplemented with 1.0 mg/L 2,4-D and BAP (MD3).

Effect of 2,4-D/BAP ratio on antioxidant activities

The antioxidant capacities of extracts of the different callus cultures and of the leaves of hawthorn used as control were determined using the DPPH and ABTS assays. Data of

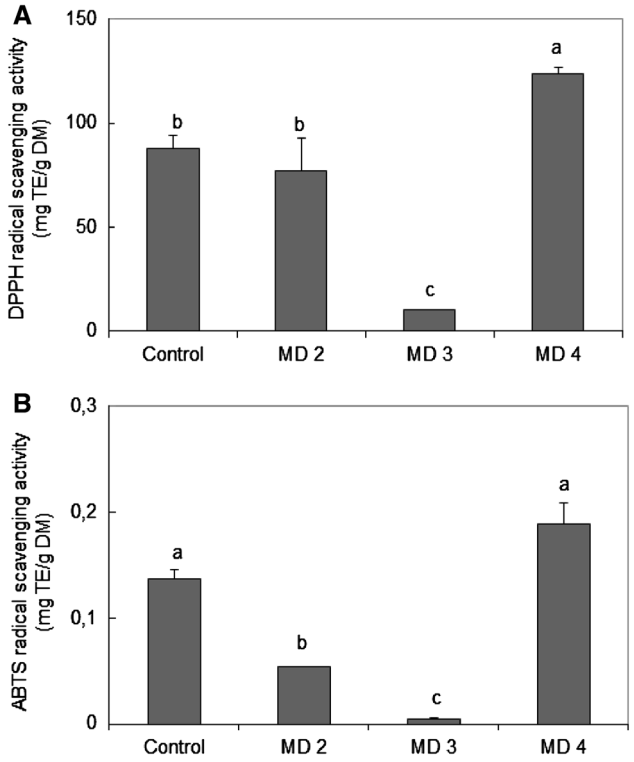


**Fig. 4** DPPH (**a**), and ABTS (**b**), radical scavenging activity of extracts of hawthorn's leaves (control) and their derived calli grown on MS media supplemented with different concentrations of 2,4-D and BAP. Means with same letters were not significantly different ($P > 0.05$). Values are mean ± SEM of three experiments. *MD1* MS + 0.5 mg/L 2,4-D + 0.5 mg/L BAP, *MD2* MS + 1.0 mg/L 2,4-D + 0.5 mg/L BAP, *MD3* MS + 1.0 mg/L 2,4-D + 1 mg/L BAP, *MD4* MS + 2.0 mg/L 2,4-D + 1.0 mg/L BAP

DPPH test of different samples presented in Fig. 4a demonstrate that extract of calli cultured on MD4 showed the best antioxidant capacity (124 ± 2.92 mg TE/g DM) by scavenging the maximum of free DPPH radical compared to the capacity of extract of calli grown on the other media that was found to vary between 10 and 77 mg TE/g DM. This activity was also significantly higher than the activity of the control extract (88 ± 5.94 mg TE/g DM). Results of ABTS radical scavenging activity illustrated in Fig. 4b were in concordance with those of antioxidant activity as evaluated by DPPH method, but here, data revealed that there was no significant difference between antioxidant capacity of extract of entire leaves (0.137 ± 0.009 mg TE/g DM) and their derived calli (0.189 ± 0.02 mg TE/g DM) on MD4.

## Discussion

The effect of using different concentrations of 2,4-D as auxin complemented with BAP as cytokinin on callus

formation, total phenolic and ascorbic acid production, and antioxidant activities in leaf explants of *C. azarolus* were investigated. Our results showed that the best callus induction as percentage and fresh weights was observed in MS medium supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L BAP. In agreement with our results, Maharik et al. (2009) reported that equal levels of cytokinin and auxin were found to induce the best callus formation; indeed, the highest values of callus fresh and dry weights were attributed to MS medium containing 1.0 mg/L 2,4-D and 1.0 mg/L kinetin (KIN) for leaf and stem explants of *Crataegus sinaica*. Similarly, Mori et al. (1994) reported that the use of MS medium containing 1.0 mg/L 2,4-D + 1.0 mg/L BAP gave the best results of callus production from leaf explants of *Fragaria ananassa cu.* Skikinari strawberry cultivar. To optimize a callus culture protocol, Al Abdallat et al. (2011) tested different types and concentrations of plant growth regulators on the callus culture of intermodal stem segments of *Crataegus aronia*. Results reported that MS medium supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L KIN produced the best average fresh weight of red colored callus. In contrast with our results, high level of BAP (3.0 mg/L) as cytokinin combined with low level of 2,4-D (1.0 mg/L) as auxin was found to stimulate the percentage, the fresh and dry weights of callus derived from internodes explants of *Catharanthus roseus* (Taha et al. 2008). The difference between our results and those obtained by Taha et al. (2008) can be due to the difference between plants and types of explants used.

From this study, total phenols produced from leaves of field-grown hawthorn and respective calli grown on different culture media were determined. Results obtained showed that the highest concentration of 2,4-D (2.0 mg/L) over BAP (1.0 mg/L) gave the maximum of phenolic contents compared to the rest of media used. These observations were supported by the findings of Mori et al. (1994) which found that a higher concentration of 2,4-D (1.0 mg/L) more than BAP (from 0.0 to 0.1 mg/L) gave good accumulation of anthocyanin in strawberry suspension cultures. Similarly, it has been found that stem callus culture of *Crataegus aronia* on MS medium supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L KIN induced a predominant red coloration confirming the enhancement of polyphenols production (Al Abdallat et al. 2011). Furthermore, Kartnig et al. (1993) demonstrated that shoot tips callus culture of *Crataegus monogyna* accumulate a high content of flavonoids and total proanthocyanidins when the MS medium was supplied with 6.0 mg/L NAA and 1.0 mg/L KIN. However, others reports (Maharik et al. 2009) showed that the addition of higher concentration of BAP (1.0 mg/L) to the culture medium more than NAA (0.5 mg/L) increased anthocyanin accumulation to 3.6 folds in stem callus culture of *Crataegus sinaica*. Similarly, El-Baz et al. (2010) suggested that the addition of higher level of BA (1.0 mg/L) as cytokinin more than NAA (0.1 mg/L) as auxin yielded the maximum of flavonoid contents in the leaf-derived calli of *Citrullus colocynthis*. Moreover, our results obtained showed that total phenolic contents in all calli cultured were significantly lower than those found in leaves used as explant source. In agreement with our results, the highest flavonoid contents in the leaf-derived calli were lower than those accumulated in leaves of the in vitro colocynth seedlings (El-Baz et al. 2010). In contrast with our results, data of Bahorun (1995) showed that phenolic contents in callus culture of *Crataegus monogyna* were similar to those observed in the original organs, and in the case of cell suspensions, these values were higher. The variation in the production of these secondary metabolites can be due to the activation or the inhibition of key enzymes phenylalanine ammonia-lyase (PAL) and/or tyrosine ammonia-lyase (TAL) which are indispensable for the biosynthesis pathway.

Concerning the effect of auxin (2,4-D)/cytokinin (BAP) ratio on the ascorbic acid contents produced in leaves of field-grown hawthorn and respective calli cultured, our results revealed that a higher concentration of 2,4-D (2.0 or 1.0 mg/L) over BAP (1.0 or 0.5 mg/L) gave the maximum of ascorbic acid contents which seemed to be the same amounts found in leaves of hawthorn. In this concern, the production of ascorbic acid and α-tocopherol in leaf tissues and callus cultures of *Centella asiatica* was studied by Norhayati et al. (2011) and they found that the amount of these two vitamins was significantly higher in the leaf-derived calli grown in MS medium containing 2.0 mg/l 2,4-D and 1.0 mg/l KIN compared to the leaf tissues. However, other reports showed higher production of vitamin C and vitamin E in leaves than in callus and cell suspension cultures of *Morinda elliptica*, (Chong et al. 2004). Furthermore, the findings of Nag et al. (2012) investigated that callus tissues of *Prosopis cineraria* cultured in MS medium supplemented with 2.0 mg/l, 2,4-D using different concentrations of glucose showed high values of ascorbic acid contents per gram of dry weight as compared to plant parts. The increasing potentialities of callus tissues to produce vitamin C may be due to the incorporation of auxin and D-glucose in the medium (Singh and Nag 1991) because the last one plays a major role as one of the precursors of ascorbic acid (Loewus and Kelly 1991). Overall, this part of research work showed that dry samples of leaf tissues of hawthorn were very rich in content of ascorbic acid. These results were in well agreement with those obtained by Tahirović et al. 2012 which demonstrated that flowers of investigated *Crataegus* L. species, dried in the drying room with ventilation at ambient temperature for 15 days, are good source of vitamin C.

In the last part of this study, the antioxidant activities of different extracts were measured using DPPH and ABTS methods and the results found confirmed those obtained in total phenols and ascorbic acid determination. In fact, extract of calli grown in culture medium supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BAP was characterized by the greatest antioxidant capacities which were similar or significantly more important than those of intact leaves. In this contest, antioxidant activities of phenolic extracts from flowers, in vitro callus, and cell suspension cultures of *Crataegus monogyna* were studied by Rakotoarison et al. 1997. They found that the scavenging activities of the cell suspension extracts were similar to those of the flowers. Furthermore, some reports studied the antioxidant actions of *Crataegus monogyna* callus cultures and showed that a high TEAC values were observed when the culture medium contained 2.0 mg/L 2,4-D and 0.5 mg/L KIN, these values were strongly influenced by the total flavonoids content (Bahorun et al. 1994, 2003). In addition, Bahri-Sahloul et al. (2014) found that ovaries calli of *Crataegus azarolus* grown on culture medium containing 2.0 mg/L 2,4-D combined with 0.5 mg/L KIN exhibited a high DPPH and ABTS radical scavenging effect. However, Grzegorczyk et al. 2007 showed a high DPPH radical scavenging effect in *Salvia officinalis* shoot cultures grown on MS medium containing a high ratio of BAP over IAA which was comparable to that of collected plants.

These biochemical analysis were also used to study the correlation between antioxidant activities and phenolic or ascorbic acid contents in different samples. Total phenols generally correlate very well with antioxidant capacities determined by the TEAC methods (Tabart et al. 2006, 2007). Numerous researchers showed a linear relationship between these two parameters (Djeridane et al. 2006; Kim et al. 2003). As shown in Table 2, four correlation coefficients were calculated between DPPH or ABTS radical scavenging activity, and the production of total phenols or ascorbic acid. Data revealed a positive correlation between total phenolic contents and DPPH or ABTS assays, with a correlation coefficient of 0.545 and 0.667 respectively, indicating the partial contribution of phenols to these antioxidant activities. These phenolic compounds possess a better scavenging effect with ABTS radical compared with DPPH free radical. A weaker positive correlation between these two methods (DPPH and ABTS) and ascorbic acid contents was demonstrated by linear regression analysis and a correlation coefficient of 0.655 and 0.300, respectively indicating only a small contribution of ascorbic acid production to antioxidant capacities of leaves and their derived calli. In fact, ascorbic acid exert an inhibitory effect on DPPH 2 times more important than on ABTS*+. Furthermore, it can be seen that DPPH radical was a little more scavenged by ascorbic acid than by phenols; whereas, ABTS radical was significantly more inhibited by phenols than by ascorbic acid. All these results suggested that phenolic compounds seemed to be a little more efficient antiradical compounds than ascorbic acid. In the same way, Bahri-Sahloul et al. (2014) demonstrated that antioxidant activity based on DPPH and ABTS assays was correlated with phenolic composition in ovaries callus culture of *Crataegus azarolus* (var. aronia) and this seemed to be strongly associated with total phenolic contents ($R_{DPPH} = 0.98$, $R_{ABTS} = 1.00$). Similarly, Dou et al. (2013) found that the total polyphenolic content in the extracts of *Crataegus pinnatifida* was significantly correlated with the IC50 value for DPPH$\dot{}$ ($r = -0.986$) and TEAC value ($r = 0.997$).

To put it briefly, equal concentration of 2,4-D and BAP (1 mg/L) supplemented to culture medium seemed to be the most suitable only for callus culture induction which gave the lowest amounts of bioactive molecules. Whereas the use of highest levels of 2,4-D (2.0 mg/L) and BAP (1.0 mg/L) was found to be the optimal combination to enhance qualitative and quantitative production of antioxidants.

**Table 2** Correlation coefficients ($R^2$) between antioxidant activities (DPPH and ABTS assays) and antioxidant content (total phenols and ascorbic acid)

| Antioxidant activities | Correlation coefficient ($R^2$) | | |
|---|---|---|---|
| | Total phenolic contents | Ascorbic acid contents | Average |
| DPPH radical scavenging activity | 0.5447 | 0.6548 | 0.5997 |
| ABTS radical scavenging activity | 0.6668 | 0.3009 | 0.4838 |
| Average | 0.6057 | 0.4775 | |

## Conclusions

From the obtained results, we can conclude that the combination between the highest level of auxin (2,4-D) over cytokinin (BAP) can help to enhance the production of total phenols, ascorbic acid, and antioxidant activities in *C. azarolus* callus cultures better than others combinations. This most suitable ratio between 2,4-D and BAP led to improve significantly the antioxidant capacities in leaf-derived calli compared with hawthorn's leaf tissues. For this reason, it can be concluded that callus culture of hawthorn under controlled conditions independently of environmental factors can be used as an alternative source of high quality of antioxidants.

**Author contribution statement** JT, CK, and JD designed all experiments of biochemical analysis performed in Belgium. LC, ML, and NKB designed in vitro

protocols, supervised, and organized the plan of study. GC performed in vitro cultures and biochemical analysis of all samples and drafted manuscript. NKB harvested plant materiel of hawthorn from the region of Ain Drahem. GC and JT made statistical analysis. GC, SZ, MIK, LC, and NKB analyzed data and interpreted results. MIK, SZ, JT, and CK carried out a critical revision of the manuscript after drafting.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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