RESEARCH ARTICLE

Effect of pre-sowing treatments and basal media on *in vitro* carob (*Ceratonia siliqua* L.) seed germination

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A fully developed protocol of *in vitro* seed germination for carob (*Ceratonia siliqua* L.) tree ranked among the most outstanding Mediterranean species is described in this study. It was found that the highest percentage (93.34%) of seed free fungi was obtained with mercuric chloride ($HgCl_2$) treatment at 1 g/L for 10 min compared to sodium hypochlorite (NaClO) at 2.6% with only 80% of disinfection. While untreated seeds showed no more than 18.11% ± 4.91% of germination, soaking carob seeds for one hour at different sulphuric acid (H_2SO_4) concentrations gave highest germination rate for seeds. Indeed, with 80% of H_2SO_4 treatment, 100% of carob seed germination was achieved in MG2 (water agar at 7 g/L + sucrose at 30 g/L) and MG3 (Murashige and Skoog half strength + Agar 7 g/L and sucrose at 30 g/L) media. Nevertheless, in MG1 medium (water with agar at 7 g/L) with 96.66% of germination gave the highest average number of leaves per plant (2.13 ± 0.30) compared to MG2 and MG3 with 1.44 ± 0.29 and 1.73 ± 0.28, respectively, showing that excess nutrients seemed to inhibit subsequent seedling growth. Furthermore, when ascorbic acid had been applied at 150 mg/L, the rates of plantlet survival and growing leaves were higher (96% ± 1.9%) than that of the control (40% ± 5.19%). However, activated charcoal treatment at 1 g/mL was more than sufficient in reducing browning and despite only providing seedling development of around 74.44% ± 4.62%, improved seedling growth with non-necrotic stems, and a better root elongation. After the transfer of the carob seedlings into the greenhouse, the survival rate was 80% and the plants grew vigorously without any phenotypic abnormalities.

Keywords: *Ceratonia siliqua*; seed; pre-sowing treatment; germination; ascorbic acid; activated charcoal; acclimatization.

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Introduction

Carob tree (*Ceratonia siliqua* L.) is an Agro-sylvo-pastoral species of the Fabaceae family and is currently considered among the most prominent Mediterranean species because of the high nutritional and economic value of its products [1-3]. Indeed, carob tree is distributed favorably in mild and dry areas with poor soils due to its physiology which allows it to develop different drought adaptation mechanisms. For this reason, it is one of the most interesting solutions to propose carob trees for the reforestation of the Mediterranean regions [4-8]. Furthermore, the principal interest of carob growers is gum derived from endosperm, as it is commonly used in food industry (sauces, mayonnaise, *etc*.) as well as in printing and cosmetic industries [4, 5, 7, 9].

Moreover, carobs' importance is expected to enhance globally due to the modern market needs and the educational efforts to promote healthy behaviors and habits. In the last few decades, many studies have demonstrated that carobs and their products are a good source of antioxidants involved in the protection against several diseases [1, 2, 6, 10]. A total of 24 polyphenol compounds found in carob fiber were actually recorded in previous research. Gallic acid in different forms dominated the profile including free gallic acid (42% of polyphenols by weight), gallotannins (29%), and methyl gallate (1%) [11]. In addition, several studies have classified carob as a source of high content in Ca, K, Mg, Na, P, Cu, Fe, Mn, and Zn [2, 10, 12]. Moreover, carob powder is a very valuable source of vitamins E, D, C, Niacin, B6, and folic acid as well as essential fatty acids [13]. Many studies have shown that carobs can promote human health and help to prevent specific chronic diseases. In particular, Custódio *et al*. (2009) demonstrated their antiproliferative and apoptotic activities against cancer cells.

In Morocco, carob tree is widely spread in the form of spontaneous or planted stands [7], and Morocco is considered to be the fourth largest producer (13.89%) after Portugal (25.46%), Italy (18.24%), and Spain (16.51%) [15]. Carob tree was first propagated by seed and later by grafting. Carob cultivars are therefore produced from random seedlings selected from local populations and subsequently planted in commercial orchards [16]. Whilst the approach including seed would not maintain clonal integrity, efficient regeneration from seed could provide information to improve the protocols used for *in vitro* regenerating adult tissues. Under normal conditions, only a few seeds germinate [17] indicating that without any pretreatment, carob germination rate will not exceed 10%. Pérez-García (2009) also pointed out that there was a high fluctuation in the germination rate (7% to 50%) of carob seeds belonging to 17 different individual trees which indicated a high degree of variability between seeds in terms of seed coat hardness. This can be attributed to genetic variation between parent plants and/or geographic regions. In fact, the physical dormancy of carob seeds is due to the impermeability of the seed coat. Carob seeds have a hard and resistant brown seed coat [19]. The coating constitutes 30% to 35% of the dry weight of the seed [20] and is impermeable to water. Carob seeds are difficult to germinate without mechanical or chemical treatments [17]. Hence, different treatments such as mechanical scarification or by soaking seed in tap or hot water, gibberellin acid (GA3) or sulfuric acid (H_2SO_4) can improve the germination rate [21]. Mechanical or acid scarification are usually used to destroy hard seed coats and raise seed germination. Most studies showed that the highest germination rate was observed when carob seeds had been soaked in sulphuric acid [21-23]. The dormancy-breaking treatments used in these situations must improve water absorption and gas exchange without altering embryo and endosperm [24]. Another problem affecting the germination and development of carob seeds is the browning of the medium. Browning is very common in the tissue culture of woody plants, which limits the growth and differentiation of explants as well as the success of tissue culture. In seed carob germination, seed coat releases brown substances or phenolic compounds to the medium, which severely affect and restrict seed germination and seedling growth. Since carob seeds are rich in phenolic compounds, their oxidation and diffusion into the medium will have a significant impact on the development of seedlings leading to medium browning as well as tissue necrosis making it difficult to cultivate. Indeed, phenolics have been confirmed to be produced by the enhanced activity of polyphenol oxidase (PPO) [25]. Besides, phenylalanine ammonia lyase (PAL) converts phenylalanine into the free phenolic substrate of PPO [26]. These two enzymes (PPO and PAL) are related to tissue browning in many woody plants, such as apple [27] and pear [28, 29]. To prevent browning, certain compounds such as ascorbic acid and activated charcoal are added to the medium [35]. Ascorbic acid is indeed an effective antioxidant that can prevent

or inhibit the process of oxidation [36], and activated charcoal is an excellent adsorbent of toxic substances [37].

Considering the request of carob tree which has increased in recent years and which requires plants with valuable characteristics, the use of *in vitro* culture is a major challenge and a promising solution to solve propagation problems in carob [33-34]. In addition to *in vitro* mass plant production, it has been shown that *in vitro* self-rooted plants are vigorous and resistant to diseases [35]. To date, *C. siliqua* seed germination has only been subject to a few studies and none of these techniques has not yet been fully completed [8, 33, 34, 36]. The aim of this study was therefore to optimize reliable methods by using several media and sulphuric acid pre-sowing treatment to improve carob germination as well as the application of ascorbic acid and activated charcoal to evaluate their effects on reducing browning of the medium with a focus on *in vitro* carob seeds germination for rapid propagation of this species.

Material and methods

Plant seed culture

Carob seeds were collected from Ras-ElMa, a homeland region of wild carob genotype, in North of Morocco (N:34°12'36.00" and W:4°00'36.00"). Random seeds of the same adult female tree were chosen to overcome the genotypic effect. Carob female plants are better carriers of pods than that of hermaphroditic plants, and the most common varieties in commercial orchards are those with female inflorescences. The collected carob seeds were rinsed with tap water before being soaked in 70% ethanol for two minutes. Afterwards, seeds were surface sterilized either with 2.6% of sodium hypochlorite (NaClO) (Sigma-Aldrich, Munich, Bavaria, Germany) with the addition of one or two drops of Tween 20 (Biotium, Fremont, CA, USA) for 20 minutes or with 1 g/L of mercuric chloride ($HgCl_2$) (Sigma-Aldrich, Munich, Bavaria, Germany) for 10 minutes to minimize microbial development in the early stages of germination. Scarification was carried out by using sulphuric acid at four different concentrations including 20%, 40%, 60%, and 80%.

In order to study the influence of medium culture composition on carob seed germination, three sterile media MG1 (water agar (Biokar Diagnostics, Pantin, French), 7 g/L), MG2 (water agar, 7 g/L + sucrose (Duchefa Biochemie, Haarlem, Netherland), 30 g/L), and MG3 (water agar, 7 g/L + sucrose, 30 g/L + Murashige and Skoog (MS) half strength of ingredients) (pH 5.7) were used and their effectiveness on carob seed germination was checked.

Activated charcoal (AC) (Sigma-Aldrich, Munich, Bavaria, Germany) and ascorbic acid (AA) (Labkem, Rungis, French) were added to the medium MG1 for the following experiments including M1 (water agar + 1 g/L of AC), M2 (water agar + 150 mg/L of AA), and M3 (water agar + soaking for one minute in 150 mg/L of AA) or used for seed soaking as mentioned below to minimize phenol secretion, which inhibits the reactivity of the seeds and remains a major problem in woody plants culture:

In order to destroy hard seed coats and increase carob seed germination, 60 minutes were applied for each treatment. To remove any trace of acid, seeds were rinsed three times in sterile distilled water (SDW), and then immersed in SDW for 24 hours before being tested for germination. Seeds were then incubated in the dark for approximately 1 week until the radicle reached about 4 mm in length. The cultures were transferred to the light and were incubated at 24 ± 1°C with 16 h photoperiod under cool white fluorescent tubes (light intensity 40 µmol/m^2.s) and were transferred to fresh medium at intervals of 4 weeks. Two months after *in vitro* seed germination, seedlings, after washing their root with tap water, were transplanted into plastic pots containing a mixture of peat and sterile sand used in the same proportion (1:1). Seedlings have been protected by jars to ensure progressive acclimatization to room

temperature. The cultures were watered once a week and maintained for 2 months in a growth chamber under 16-photoperiod and a temperature of 24 ± 1°C.

Statistical analysis

In this investigation, experiments were performed on the basis of a completely randomized factorial design. All experiments have been repeated three times with a minimum of 30 replicates per treatment. Data were analyzed by Pearson's chi-squared test and Fisher's exact test in case of categorical variables or the analysis of variance (ANOVA) method in case of quantitative variables. The significance of differences among the mean values was examined using Duncan's multiple range test (DMRT) at 5% using the IBM SPSS Statistics 22 software (IBM, Armonk, NY, USA).

Results and discussion

***In vitro* carob seed germination and development of aseptic seedlings**

The elimination of microorganisms in plant *in vitro* culture is one of the challenges for successful micropropagation, especially in woody plants. Due to endogenous contamination, both the initiation and growth stages of *in vitro* culture pose serious problems [37]. For most plant tissue cultures, losses due to microorganism contaminants may often be up to 15% [38]. Therefore, the first experiment in this investigation was performed to determine the effects of two different sterilizing agents, 1% of $HgCl_2$ and 2.6% of NaClO, on surface sterile carob seeds which were cultivated on three media, MG1, MG2, and MG3. Infection and success rates were calculated after one month of cultivation. The results showed that treatment with $HgCl_2$ for 10 min was more effective as a disinfectant for the establishment of the largest number of surface sterile seeds as it gave a higher result with 93.34% of seed free fungi than that of NaClO which showed the lowest disinfection frequency of 80% ($P<0.05$). However, the two sterilizing agents used had a significant effect on low bacterial proliferation since bacterial contamination was reduced to 2.22% for both treatments.

Carob seeds are characterized by tough envelopes making their germination difficult or even impossible. Scarification with sulphuric acid was used to improve carob seed germination by softening the seed coats while rendering them permeable to water, enabling the seed to germinate. Many previous published studies have used sulphuric acid for scarification [34, 39, 40]. To find suitable concentrations of sulphuric acid, four different concentrations ranging from 20-80% were applied with each application for 60 minutes. The germination of a seed begins with the absorption of water and is completed when the radicle protrudes from the seed coat. Moreover, in this investigation, we used three media (MG1, MG2, and MG3) to emphasize the importance of medium composition as well as sucrose rich medium in improving carob seed germination and seedling growth and whether nutrients brought into the culture medium interferes with or inhibits seedling development. Indeed, the germination rate of carob seeds was improved significantly when soaking in sulfuric acid and we noticed that the germination rate was increased proportionally to the concentrations of sulphuric acid used during this treatment (Table 1). With 80% of sulphuric acid, 100% of carob seeds germination is achieved without causing damage to the embryos in the seeds whereas the untreated seeds showed no more than 18.11 ± 4.91% of germination in MG2 medium ($P<0.01$), which confirmed the existence of the tough seed coat in the carob seeds and the fact that sulfuric acid treatment seemed to be effective for their softening. We can then conclude that there is a very significant relationship between the pre-sowing treatment and germination rates ($P<0.01$). These findings are similar to those of Cavallaro *et al*. (2016) and Gharnit and Ennabili (2010) [39, 40]. However, it is important to note that the majority of the germination rates reported in the literatures have never reached 100%. Lamloum (2016)

Table 1. Effect of the medium and the scarification on *in vitro* carob seed germination.

Medium	Control	H_2SO_4 (20%)	H_2SO_4 (40%)	H_2SO_4 (60%)	H_2SO_4 (80%)
MG1	13.33 ± 3.60%	34.44 ± 5.05%	37.42 ± 3.04%	43.33 ± 5.25%	96.66 ± 1.90%
MG2	18.11 ± 4.91%	32.22 ± 4.95%	36.66 ± 5.11%	47.77 ± 5.29%	100.00 ± 0.00%
MG3	15.55 ± 3.84%	28.88 ± 4.80%	40.00 ± 5.19%	42.33 ± 5.15%	100.00 ± 0.00%

Table 2. Effect of the medium on growing carob plantlets from germination (Leaves growing).

Medium	H_2SO_4 (20%)	H_2SO_4 (40%)	H_2SO_4 (60%)	H_2SO_4 (80%)
MG1	1.33 ± 0.27	1.70 ± 0.31	2.00 ± 0.18	2.13 ± 0.30
MG2	1.12 ± 0.23	1.20 ± 0.27	1.00 ± 0.25	1.44 ± 0.29
MG3	0.60 ± 0.15	1.12 ± 0.29	1.77 ± 0.31	1.73 ± 0.28

announced that soaking seed in concentrated sulphuric acid for 15 min gave a germination rate about 90.78% [41]. In the same way, Bostan and Kilic (2014) and Pérez-García (2009) recorded their highest germination rates of 86% and 88.89%, respectively when carob seeds were soaked in concentrated sulphuric acid for 30 min [22, 38]. The success rate in this study can be related to the 1-hour treatment time used where sulfuric acid acts more on the softening of the seed coat. Furthermore, in this case, it turns out that sucrose-rich media such as MG2 and MG3 are more favorable to stimulate germination regardless of mineral contents (Table 1). Seeing that 100% of carob seed germination was achieved in MG2 and MG3 media, while only 96.66% of germination was reported in MG1 medium ($P<0.05$), which suggested a significant medium effect on germination. Our results contradict those of Gharnit and Ennabili (2010) who affirmed that the best germination rate was obtained in water agar compared to half strength MS medium with sucrose or sucrose-water mixture used in their investigation [40]. Nevertheless, the single most striking observation emerging from the data comparison was that MG1 medium with water agar gave the highest average number of leaves per sprouted plant (2 ± 0.18) as illustrated by Table 2 and Figure 1a. In fact, a one-way ANOVA analysis revealed that the medium and pre-sowing treatment had a significant effect on leaves number per shoot (Table 2). Indeed, the number of leaves per sprouted plant in MG1 medium was very high and ranged between 1.33 ± 0.27 to 2.13 ± 0.30. However, in MG2 and MG3 media, the highest numbers were recorded for H_2SO_4 (80%) with 1.44 ± 0.29 and 1.73 ± 0.28, respectively (Table 2 and Figure 1b). MG1 medium seemed sufficient to trigger seedling growth and a medium rich in nutrients as MG2 seemed to inhibit it. Since the seeds have accumulated all the reserves necessary for their development as an initial energy source in the heterotrophic growth phase of germination and seedling establishment, the excess of nutrients brought into the culture medium may interfere with or even inhibit seedling development.

During germination and seedling development, a strong secretion of phenols has been observed, which seems to prevent seed germination as well as the development of seedlings to make seeds necrotic and die. Indeed, the diffusion of these phenolic compounds was observed in all tested media and more especially in MG2 medium containing agar and sucrose, which led to epicotyls and root of the seeds browning (Figure 1c). In fact, the use of sulphuric acid is mainly intended to soften the seed coat, which is considered rich in polyphenols by several authors [43]. Softening of the seed coat could be followed by the release of phenols, and therefore, control seeds keeping a high dose of polyphenols, which could affect the germination of the seed and its subsequent development leading to the

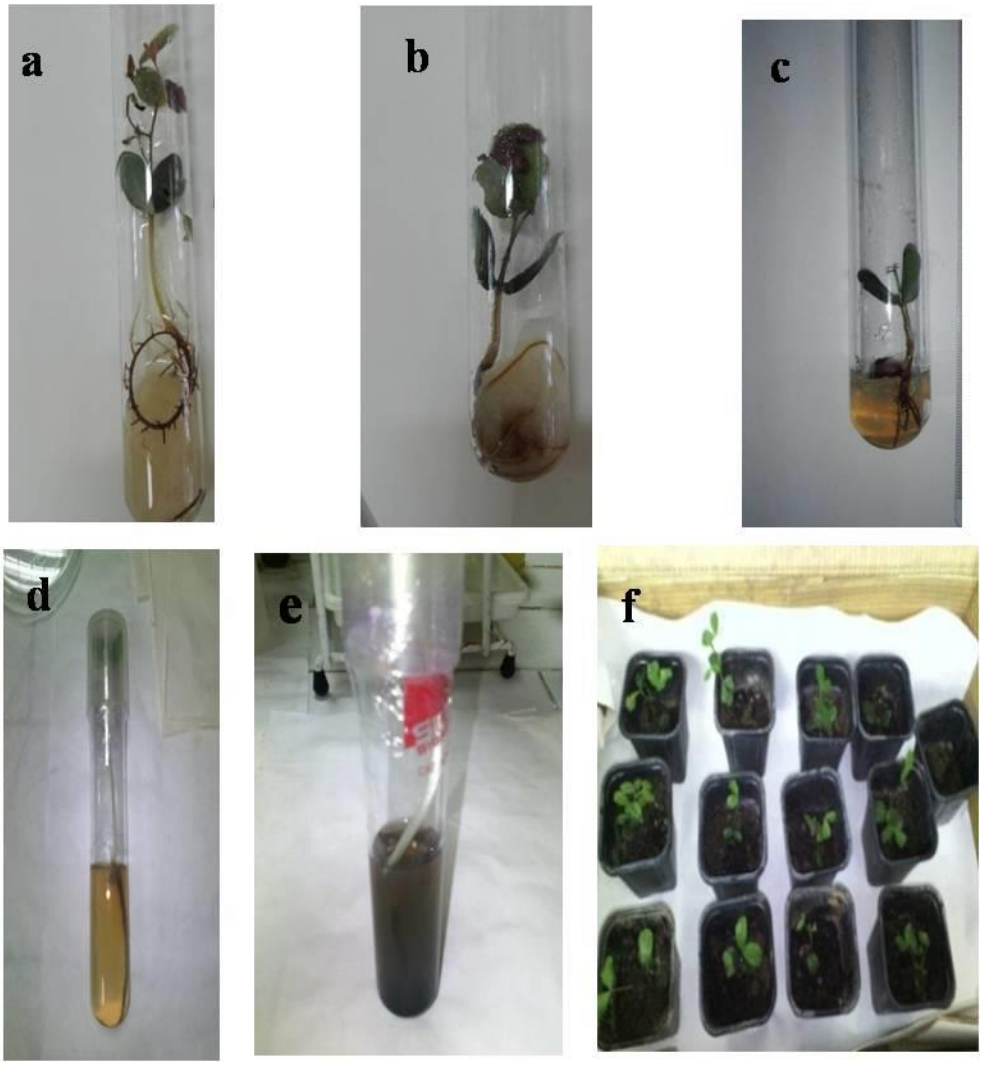

Figure 1. Micro-propagated seedlings of carob tree before and after transplantation. **a:** *in vitro* carob seedling development in MG1 medium. **b:** *in vitro* carob plantlet development in MG2 medium. **c:** necrotic brown seedling in MG2 medium. **d:** *in vitro* carob plantlet development in M1 medium with AA. **e:** *in vitro* carob plantlet development in M3 with AC. **f:** acclimatized carob plants 8 weeks after *ex vitro* transplantation.

inhibition of growth and very brown medium [44]. This tissue browning was resolved by a regular transfer of seedlings to a fresh medium, where a gradual cessation of phenol secretions was noted. Previous works on this species have also published similar findings [45, 46]. To prevent browning of the culture media, activated charcoal (AC) as well as ascorbic acid (AA) which were known to reduce browning and necrotic problems of tissue and seed germination were applied during the initiation phase after 80% of sulfuric acid treatment [30]. After 15 days of culture on medium with water agar containing either 1 g/L of AC (M1), or 150 mg/L of AA (M2), or with one minute soaking in 150 mg/L of AA (M3), the intensity of phenols secretion was reduced as the culture medium remains clear and transparent. The rates of survival and growing leaves were higher (96 ± 1.9%) when AA was applied than that of the control in MG1 with only 40 ± 5.19% of growing leaves ($P<0.05$). Apart from its antioxidant properties, ascorbic acid is also involved in cell division and elongation, allowing seedlings to grow rapidly [47]. Furthermore, a one-minute soak in AA (150 mg/L) prevented browning and helped seedlings

to grow at 91 ± 3.02% ($P<0.05$). Despite the high growth rate obtained with ascorbic acid, this antioxidant product had become ineffective in the case of high phenol secretion, and the concentration used (150 mg/L) had not been sufficient enough in the long term to reduce browning (Figure 1d). The addition of AC at the concentration of 1 g/L also counteracted browning and provided seedling growth of around 74.44 ± 4.62%. Although, statistically significant differences were observed among the experimental and control groups, seedlings growing in M1 medium showed better root growth with non-necrotic stems and AC at the concentration of 1 mg/L was sufficient to reduce browning (Figure 1e). Based on this finding, it can be suggested that AC, due to its strong chelating effect, has provided encouraging results in terms of reducing phenolic diffusion, and therefore, improved seedling growth, as AC increases the stability of phenols, and therefore, permits less oxidation. Activated charcoal seems to be an important part of carob tissue culture medium. Most of the previous studies focused on the effects of scarification types and the genotypes on carob seed germination. In this study, along with 80% of sulphuric acid scarification applied for 1 h followed by a soaking in distilled water for 24 h, we investigated the effects of medium composition and emphasized the importance of sucrose rich media on carob seed germination and activated charcoal on improving seedling growth.

Acclimatization

Two months after germination, seedlings were transferred to plastic pots. After eight weeks, acclimatization was successful in 80% of the potted plantlets and the size of the plantlets ranged from 5 to 12 cm (Figure 1f). The loss of 20% of acclimatized plants might be attributed to the fact that the long-rooted plants were easily damaged during the transplantation process [48], and that damaged roots would promote the emergence of infected areas caused by pathogens, such as fungi in this case, affecting the survival of plants. An antifungal treatment seems to be necessary as well as further care during the transplant process for the next acclimatization in order to save a maximum of seedlings. The acclimatized plants were successfully adapted to *in vivo* conditions, and then placed in a greenhouse where they grew vigorously without any phenotypic abnormalities.

Conclusion

The present study was conducted to increase carob seeds germination from Ras-ElMa, a homeland region of wild carob genotype in north part of Morocco, and to determine the best treatment and media for *in vitro* seed germination. These results showed the highest significant contamination-free seed rate (96.66%) with $HgCl_2$ at the concentration of 1 g/L for 10 min. In addition, a treatment with 80% of sulphuric acid for one hour followed by 24 h of soaking in SDW achieved 100% of carob seed germination in sucrose-rich media (MG2 and MG3) which promoted germination regardless mineral contents. Otherwise, the MG1 medium gave the highest average number of leaves per plant (2.13 ± 0.30). Furthermore, using activated charcoal at the concentration of 1 mg/L helped eradication of germination browning during seed growth with increased root growth and without necrotic stems. The originality of this protocol was to study the effect of medium composition in parallel with the scarification and highlighting the importance of activated charcoal in the improvement of the seedling growth. This detailed protocol on the germination and acclimatization of carob seeds could be used for mass propagation of carob plants for their economic benefit, reforestation of Mediterranean regions, and commercial cultivation.

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