

***In Vitro* Shoot Formation on Sugarcane (*Saccharum officinarum* L.)
Callus as Affected by Benzyladenine Concentrations**

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ABSTRACT

In vitro regeneration of sugarcane (*Saccharum officinarum* L.) clones can support breeding program of sugarcane. This research was conducted to study the effect of benzyladenine on *in vitro* shoot formation from sugarcane callus. Leafrolls were cultured for 8 weeks on callus induction medium containing MS salts, 30 g L⁻¹ sucrose, 150 ml L⁻¹ coconut water, 100 mg L⁻¹ myo-inositol, 0.1 mg L⁻¹ thiamine-HCl, 0.5 mg L⁻¹ pyridoxine-HCl, 0.5 mg L⁻¹ nicotinic acid, 2 mg L⁻¹ glycine, and 3 mg L⁻¹ 2,4-D. Callus was then subjected to different concentrations of benzyladenine (BA) (0, 0.5, 1, 2, and 2.5 mg L⁻¹) contained in MS media. The experiment showed that after 8 weeks in culture 2 and 2.5 mg L⁻¹ BA led to the highest percentage of shoot formation (100%). The experiment also showed that addition of BA caused an increase in percentage of shoot formation, number of shoot per callus clumps, and average shoot length. In the range of 0-2.5 mg L⁻¹, the higher the concentrations of BA the more shoots and the longer shoots were produced. Highest number of shoots was recorded at BA 2.5 mg L⁻¹ (36.4 shoots per callus clump) and highest average shoot length was obtained at 2 and 2.5 mg L⁻¹ BA, i.e. 2.25 and 2.3 cm respectively. The shoot formation system was then applied to 12 sugarcane genotypes, resulting in statistically different response and producing substantial number of shoots, ranging from 29 to 41.33 shoots per clump.

Keywords: *In vitro*, sugarcane, leafrolls, benzyladenine, shoot formation

ABSTRAK

Regenerasi *in vitro* tanaman tebu (*Saccharum officinarum* L.) dibutuhkan untuk mendukung program pemuliaan tanaman tebu. Penelitian ini bertujuan untuk mengetahui pengaruh benziladenin (BA) terhadap pembentukan tunas *in vitro* dari kalus tanaman tebu. Eksplan berupa gulungan daun muda dikulturkan selama 8 minggu pada media induksi kalus yang mengandung garam dari MS, 30 g L⁻¹ sukrosa, 150 ml L⁻¹ air kelapa, 100 mg L⁻¹ myo-inositol, 0.1 mg L⁻¹ tiamin-HCl, 0.5 mg L⁻¹ piridoksin-HCl, 0.5 mg L⁻¹ asam nikotinat, 2 mg L⁻¹ glisin, dan 3 mg L⁻¹ 2,4-D. Kalus kemudian diperlakukan dengan beberapa konsentrasi BA (0, 0.5, 1, 2, and 2.5 mg L⁻¹) dalam media MS. Hasil percobaan menunjukkan bahwa setelah 8 minggu perlakuan 2 dan 2.5 mg L⁻¹ BA menghasilkan persen pembentukan tunas tertinggi, yaitu 100%. Hasil percobaan juga menunjukkan bahwa BA menyebabkan peningkatan persentase pembentukan tunas, jumlah tunas per kalus, dan panjang tunas. Pada kisaran 0-2.5 mg L⁻¹, semakin tinggi konsentrasi BA, semakin banyak dan semakin panjang tunas yang dihasilkan. Jumlah tunas terbanyak dihasilkan dengan pemberian 2.5 mg L⁻¹ BA (36.4 tunas per kalus) dan tunas terpanjang dihasilkan dari perlakuan 2 dan 2.5 mg L⁻¹ BA, yaitu 2.25 dan 2.3 cm. Prosedur pembentukan tunas *in vitro* tersebut lalu diaplikasikan untuk memperbanyak tunas secara *in vitro* 12 genotipe tanaman tebu. Hasilnya adalah bahwa respon pembentukan tunas dari genotipe-genotipe tersebut secara statistik berbeda nyata dan menghasilkan tunas dalam jumlah banyak, yaitu antara 29 to 41.33 tunas per kalus.

Kata kunci: *in vitro*, tebu, gulungan daun, benziladenin, pembentukan tunas

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), a member of family of Graminae and genus of Saccharum, is an important crop in tropical and subtropical regions. It is cultivated as a cash crop in virtually 60 countries located in five continents (Zucchi *et al.*, 2002). Sugarcane accounts for approximately

80% of raw material for the production of sugar in the world (Illovo Sugar, 2009). The ten biggest producers of sugar in the world (in million tons) are Brazil (38.6), India (16.3), EU (14.9), China (13.6), Thailand (7.7), USA (6.9), Mexico (5.8), South Africa (5.3), Australia (4.80), and Pakistan (4.2) (Illovo Sugar, 2009). In 2011, Indonesia produced 2.2 million tons of sugar while the total demand was 2.7 million tons (The Jakarta Post, 2012). In 2012, this country was predicted to produce 2.68 million tons of sugar (IAARD, 2012) while the demand was predicted 2.8 million tons (The

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Jakarta Post, 2012). Therefore, to be self-sufficient in sugar, Indonesia has to increase sugar production.

There are three ways of increasing sugar production, i.e. by increasing plantation areas, cane productivity, and sugar recovery. The most effective and cheapest way of increasing sugar production is by rising sugar recovery (Pakpahan, 2009), and to do that one way is by growing sugarcane clones that have high sugar recovery. A breeding program supported by plant tissue culture could pave the way to produce high sugar recovery clone of sugarcane.

Plant tissue culture of sugarcane has been reported by several researchers (Gandonou *et al.*, 2005; Khan and Khatri, 2006; Khan *et al.*, 2006; Ali *et al.*, 2008a, 2008b; Khan *et al.*, 2008; Mordocco *et al.*, 2008; Khan *et al.*, 2009; Ali *et al.*, 2010; Raza *et al.*, 2010). Three patterns of plantlet formation were observed i.e. axillary branching, somatic embryogenesis, and organogenesis. In axillary branching, shoot tips were used as explants and induced to form multiple shoots on benzyladenine (BA)-containing media (Ali *et al.*, 2008a; Khan *et al.*, 2006). Each shoot was then promoted to produce roots on media containing indole butyric acid (IBA) and naphthaleneacetic acid (NAA) (Ali *et al.*, 2008a). In somatic embryogenesis, young leaves were used as explants and promoted to form embryogenic callus on 2,4-dichlorophenoxyacetic acid (2,4-D)-containing media and the callus was then subcultured on media supplemented with lower concentration of 2,4-D to induce somatic embryo formation. Plant regeneration was achieved by transferring the somatic embryo on media supplemented with kinetin, IAA, and IBA (Khan and Khatri, 2006). In organogenesis, leaf explants were cultured on media containing 2,4-D to induce formation of callus that was then transferred to media containing BA or BA and kinetin to induce shoots (Ali *et al.*, 2008b).

Previous experiments also demonstrated that genotype influenced sugarcane regeneration. Therefore, even though successful *in vitro* regeneration of sugarcane has been reported by many researchers, experiments to establish tissue culture protocol should be conducted for each genotype. In this paper we reported the effect of BA on *in vitro* shoot formation on sugarcane callus and we demonstrated that genotypes could affect shoot regeneration *in vitro*. This research was part of an effort to establish protocol of *in vitro* regeneration of sugarcane which can be applied to a wide range of genotypes.

MATERIALS AND METHODS

Plant Materials and Sterilization

Explants were leafrolls excised from shoot tip of sugarcane (*Saccharum officinarum* L.) generously provided by PT Gunung Madu Plantations, Lampung. Twelve clones of sugarcane were used in this experiment, i.e. RGM 97-10120; RGM 97-8752; RGM 97-8837; RGM 99-599; RGM 99-515; RGM 2000-612; RGM 2000-867; RGM 2000-477; RGM 2000-1050; RGM 2000-857; RGM 2000-469; and GM 19. Shoot tips cutted from 4 month old sugarcane plants

(5 cm in length) were washed using tap water, then surface sterilized by quick-dipping into 75% alcohol and shaking in 1.57% sodium hypochlorite for 10 minutes, rinsing with sterile distilled water three times, shaking in 0.26% sodium hypochlorite for 5 minutes and followed by rinsing with sterile distilled water.

Callus Induction

Shoot tips were aseptically cutted to form leafrolls approximately 2 mm thick. The explants were cultured on callus induction (CI) medium to induce callus. The CI medium consists of MS salts (Murashige and Skoog, 1962), 30 g L⁻¹ sucrose, 150 ml L⁻¹ coconut water, 100 mg L⁻¹ myo-inositol, 0.1 mg L⁻¹ thiamine-HCl, 0.5 mg L⁻¹ pyridoxine-HCl, 0.5 mg L⁻¹ nicotinic acid, 2 mg L⁻¹ glycine, and 3 mg L⁻¹ 2,4-D. The cultures were incubated for 4 weeks in the dark in a culture room of 25 °C ± 2 °C and subcultured on the same medium for another 4 weeks.

Shoot Induction

This study consisted of two experiments. In the first experiment, callus of sugarcane clone RGM 97-10120 from CI medium was transferred to shoot induction (SI) medium which consists of MS salts (Murashige and Skoog, 1962), 30 g L⁻¹ sucrose, 100 mg L⁻¹ myo-inositol, 0.1 mg L⁻¹ thiamine-HCl, 0.5 mg L⁻¹ pyridoxine-HCl, 0.5 mg L⁻¹ nicotinic acid, 2 mg L⁻¹ glycine, and different concentrations of BA (0, 0.5, 1, 2, and 2.5 mg L⁻¹) as treatments. In the second experiment, callus was transferred to SI medium containing the best concentration of BA obtained in the first experiment. The cultures were put in a culture room provided with fluorescent lamps of approximately 1,000 lux. The temperature was maintained at 25 °C ± 2 °C.

Experimental Design and Statistical Analysis

The first experiment consisted of 5 treatments, i.e. different concentrations of BA (0, 0.5, 1, 2, and 2.5 mg L⁻¹) in SI medium and the second experiment comprised of 12 genotypes of sugarcane as treatments. The two experiments were arranged in a completely randomized design with three replicates, the experimental unit being 5 bottles of media with one clump of callus in the first experiment and 3 bottles of media with one clump of callus in the second experiment. Data was subjected to analysis of variance and comparison of treatment means was employed using least significant difference.

RESULTS AND DISCUSSION

Leaf rolls were used as explants (Figure 1A). After 2 weeks in callus-inducing (CI) medium, callus started to appear along the cut surface of the explants (Figure 1B). After 4-6 weeks in culture, the callus was transferred to the same medium for 4-6 weeks to let the callus proliferate (Figure 1C, D). In the first experiment, callus was then

transferred to shoot-inducing (SI) medium containing different concentrations of BA. Shoots were formed after 2 weeks in SI medium (Figure 2).

The first experiment showed that addition of BA caused an increase in percentage of shoot formation (Figure 3), number of shoot per callus clump (Figure 4), and average shoot length (Figure 5). An increase in BA concentrations

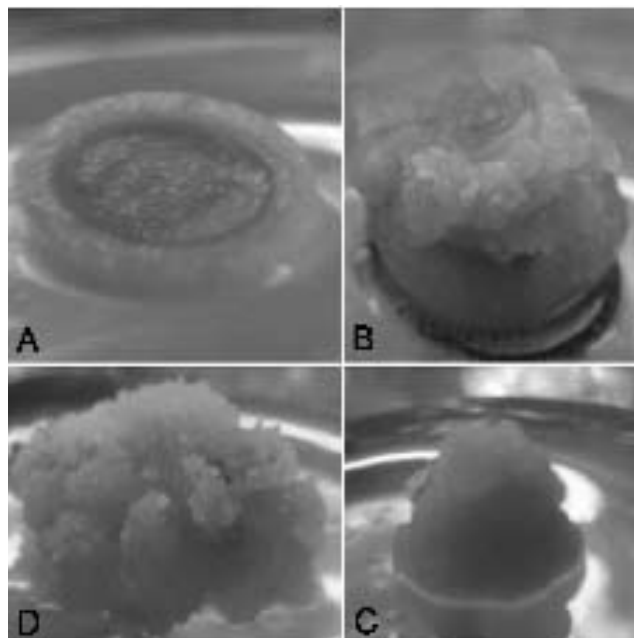


Figure 1. Leafrolls of sugarcane shoot tips were used as explants and cultured on callus induction medium (A). After 4 weeks in culture, callus proliferates along the cut surface of explants (B). A clump of callus taken from B was cultured on callus induction medium (C) and after 6 weeks in culture callus proliferated (D)

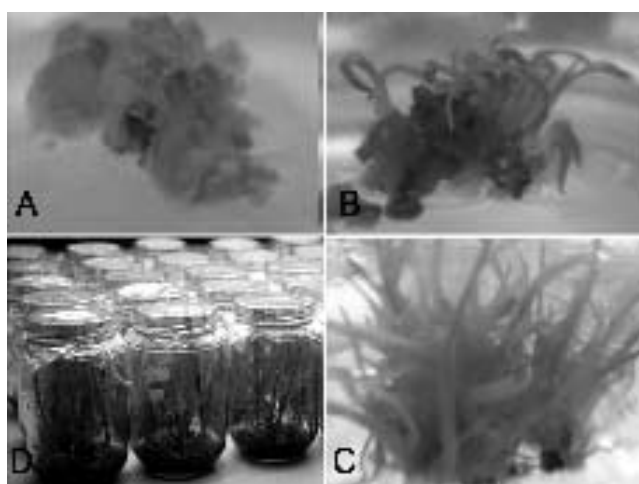


Figure 2. Callus was cultured on shoot induction medium for 4 weeks ; shoot buds appeared on the surface of callus (A). After 6 weeks in culture shoots elongated (B). After 8 weeks in culture more shoots were formed (C). Shoots were cultured on medium containing activated charcoal to induce shoot elongation and root initiation (D). These shoots were ready for acclimatization

also caused an increase in those three parameters (Figure 3-5). BA of 2 and 2.5 mg L⁻¹ led to the highest percentage of shoot formation (100%) (Figure 3). In the range of 0-2.5 mg L⁻¹, the higher the concentrations of BA the more and the longer shoots were produced (Figure 4-6). Highest number of shoots was recorded at BA 2.5 mg L⁻¹ (36.4 shoots per callus clump) and highest average shoot length was obtained at BA 2 and 2.5 mg L⁻¹, i.e. 2.25 and 2,3 cm respectively (Figure 4-6).

It is well documented that shoot induction *in vitro* is usually achieved by addition of cytokinin in culture medium. In this experiment, shoot formation was also achieved by addition of BA in shoot induction medium. Other researchers has also demonstrated that BA promoted shoot formation in various plants such as *Sarcostemma brevistigma* (Thomas and Shankar, 2009), *Melothria maderaspatana* (Baskaran *et al.*, 2009), rice (Yin *et al.*, 2008), sorghum (Pola *et al.*, 2007), and corn (Sairam *et al.*, 2003). In sugarcane, BA has also been shown to have promotive effects on shoot formation (Gandonou *et al.*, 2005; Ali *et al.*, 2008a; 2008b; Khan *et al.*, 2009).

Increase in BA concentrations led to increase in number of shoots, a phenomenon that also reported by other researchers on tissue culture of other plants. Increase in BA concentration from 2 mg L⁻¹ to 2.5 mg L⁻¹ caused an increase

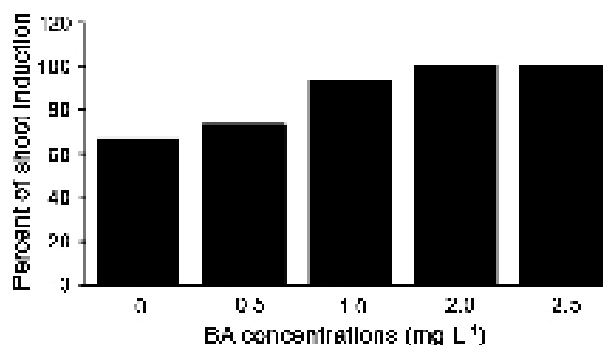


Figure 3. Effects of benzyladenine (BA) concentrations on percentage of shoot induction of sugarcane callus in vitro after 8 weeks in culture

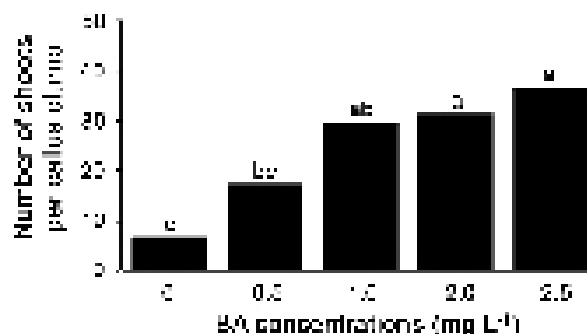


Figure 4. Effects of benzyladenine (BA) concentrations on number of shoots of sugarcane callus in vitro after 8 weeks in culture. Bars accompanied by the same letter are not significantly different based on least significant difference (LSD 0.05)

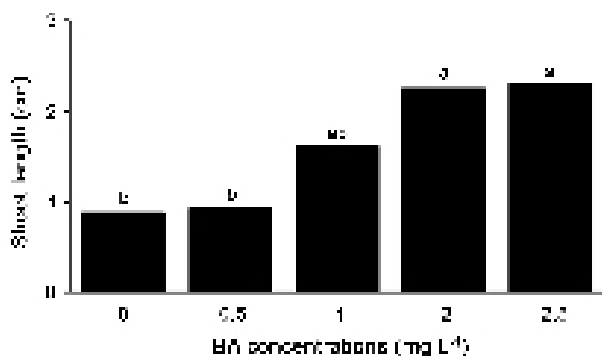


Figure 5. Effects of benzyladenine (BA) concentrations on length of shoots on sugarcane callus in vitro after 8 weeks in culture. Bars accompanied by the same letter are not significantly different based on least significant difference (LSD 0.05)



Figure 6. Effects of benzyladenine (BA) concentrations on shoot formation on sugarcane callus in vitro after 8 weeks in culture: BA 0.5 mg L⁻¹ (A), BA 1 mg L⁻¹ (B), and BA 2 mg L⁻¹ (C)

in number of shoot, even though it was not statistically significant, suggesting that BA at 2 mg L⁻¹ was not maximum value for shoot induction. Even though adequate number of shoots induced in media containing 2.5 mg L⁻¹ BA exhibited vigorous growth, further experiment needs to be done to get more shoots and to find out the maximum concentration of BA for shoot induction.

A classic paper written by Skoog and Miller (1957) is still used by plant tissue culturists as a basis in regenerating plants *in vitro*. According to the paper, *in vitro* plant regeneration is dependent upon combination of phytohormone auxin and cytokinin. High auxin to cytokinin

ratio promotes root formation, while low auxin to cytokinin ratio induces shoot formation. Balanced concentration of auxin and cytokinin induces callus formation. In our report here, callus induction in sugarcane was attained using 3 mg L⁻¹ 2,4-D, suggesting that balanced concentration of auxin and cytokinin was achieved.

When moved to hormone-free-medium, 66.66% of calluses produced shoots (Figure 3), indicating that even without addition of cytokinin the auxin to cytokinin ratio favored the formation of shoots. Addition of 0.5-2.5 mg L⁻¹ BA, or decreasing auxin to cytokinin ratio, increased the percentage of shoot formation up to 100% and increased the number of shoot per callus clump; the highest number of shoots (36.4 shoots per callus clump) was obtained using 2.5 mg L⁻¹ BA (Figure 4). This concentration of BA was then used for the second experiment to know the effects of genotypes of shoot regeneration.

The second experiment showed that genotypes significantly affected shoot regeneration as indicated by number of shoots (Figure 7). The most responsive genotype was RGM 99-599 that produced 41.33 shoots per clump while the least responsive one was RGM 97-8837 that produced 29 shoots per clump.

Callus and shoot formation has been achieved in this experiment from highly specialized tissue, i.e. young sugarcane leaves (leaf rolls). The shoots were formed indirectly following the callus formation. George (1996) described progressive steps in the capacity of a cell to become morphogenic. The highly differentiated cells undergo dedifferentiation to become callus, which consist of undifferentiated, unorganized cells. This state is called ground state. Upon exposure to stimuli, usually a hormone, the cells become competent cells, which are those having capacity to undergo certain pathway of morphogenesis. The competent cells then get into determined state, in which the cells become committed to follow certain pathway of morphogenesis. In practice, it is difficult to make a separation between competent state and determined state.

In this research, shoots could be produced after callus was transferred to hormone-free medium (Figure 3 and 4), indicating that hormone-free medium was a stimulus for the callus to get into determined state to become shoots and this

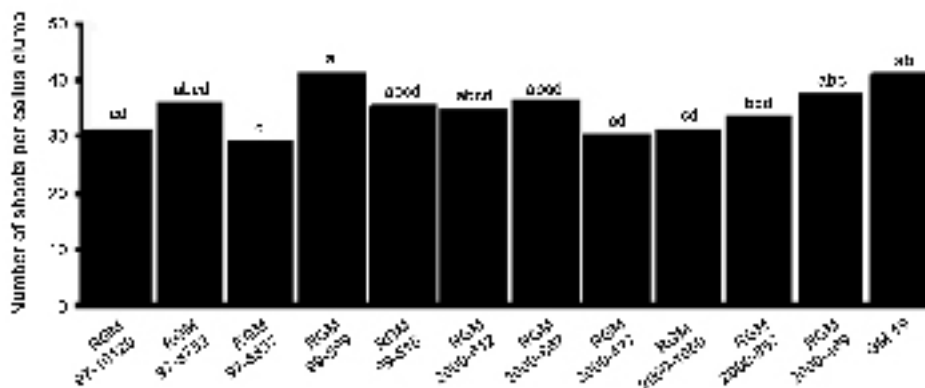


Figure 7. Effects of genotypes on number of shoots of sugarcane callus in vitro after 8 weeks in culture. Bars accompanied by the same letter are not significantly different based on least significant difference (LSD 0.05)

could be enhanced by addition of BA. Based on auxin to cytokinin ratio concept put forward by Skoog and Miller (1957), culture media devoid of auxin and that with addition of BA suggested condition of low auxin to cytokinin ratio. Therefore, this resulted in shoot formation, a determined state.

Another possibility was that the determined state had been achieved before the callus was moved to hormone-free medium or to BA-containing medium. In other words, the callus capable of becoming shoots was not the determined state, but callus capable of turning into somatic embryo was the determined state. In this case, 2,4-D acted as stimulus to induce callus to become embryogenic and when subcultured to hormone-free or cytokinin-containing medium, the callus developed into somatic embryos, underwent maturation, and finally germinated to form shoots.

Sugarcane regeneration via somatic embryogenesis has been reported by some researchers (Ahloowalia and Maretzki, 1983; Snyman *et al.*, 2000; Khalil, 2002; Desai *et al.*, 2004; Gill *et al.*, 2004; Khan and Khatri, 2006). In those reports, embryogenic callus was induced in medium containing auxin alone or in combination with cytokinin, then its development into somatic embryo and shoot regeneration was achieved when the embryogenic callus was subcultured on medium containing lower level of auxin or no auxin and/or with cytokinin. Similar pattern of shoot regeneration via somatic embryogenesis has also been reported in other graminiae species such as rice (Aly *et al.*, 1998; Ilahi *et al.*, 2005; Lestari and Yunita, 2008; Vega *et al.*, 2009).

The result of the first experiment, that employed only one sugarcane genotype, had been applied to the second experiment covering 12 sugarcane genotypes. The second experiment showed that genotypes significantly affected shoot formation. Genotype-dependent response to in vitro culture in sugarcane has also been reported by other researchers (Gill *et al.*, 2004; Gandonou *et al.*, 2005; Raza *et al.*, 2010). Even though genotypes influenced in vitro regenerability, from a practical point of view 29 to 41.33 shoots per clump produced in our experiment should be efficient enough for sugarcane micropropagation. Rooted shoots of sugarcane have been available and ready to be acclimatized (Figure 2D).

CONCLUSIONS

A substantial number of sugarcane shoots was produced in vitro by culturing leafrolls of sugarcane on MS medium supplemented with 3 mg L⁻¹ of 2,4-D for 8 weeks to induce callus formation and culturing the callus to MS medium supplemented with 2.5 mg L⁻¹ BA for 8 weeks to promote shoot formation. This procedure produced 100% response of shoot formation with 36.4 shoots per callus clump. This shoot formation protocol proved to be effective for inducing shoot multiplication in 11 other sugarcane genotypes, leading to 29 to 41.33 shoots per callus clump.

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