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GLOBULAR EMBRYO REGENERATION FROM CALLUS IN THE TEA PLANT (*CAMELLIA SINENSIS* [L.] O. KUNTZE)

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ABSTRACT
The techniques used in tea plant production remain insufficient. Hence, in the study, MS media were tested with IAA and KIN to define the effects of plant growth regulators, as well as to find the proper doses and different carbon sources that are effective on somatic embryo regeneration in the tea plant (*Camellia sinensis* (L.) O. Kuntze) *in vitro*. To examine the effects of culture conditions on regeneration, cultures were kept in dark and photoperiod conditions. As a result of the study, sucrose has been determined as the most effective carbon source, while the best embryonic callus regeneration including globular embryos has been achieved at 20 mg L^{-1} (80%) and 30 mg L^{-1} (76%) sucrose doses. Although there has been no significant difference between the effects of IAA and KIN, it has been determined that embryogenic callus regeneration rates increased in higher doses of them. In the experiment on culture conditions, photoperiod conditions (83.3%) were more effective than dark environment conditions (53.3%) in terms of embryogenic callus regeneration.

***Keywords*:** *Camellia sinensis (L.) O. Kuntze, Embryogenic callus, Carbon sources, Growth regulators*

INTRODUCTION
Tea (*Camellia sinensis* (L.) O. Kuntze) is an important agricultural plant from the tea family (Theaceae) that grows in humid climates and whose leaves and buds are used to produce beverages. It is one of the most popular beverages consumed worldwide, both hot and cold. Although its homeland is South and Southeast Asia, it is grown in about 40 countries around the world (in countries with tropical and subtropical climate conditions). The species grown for agricultural purposes have an evergreen color that looks like a small tree under 2 m. In wild species, it can reach 9 m in length. It has a strong main root. (Baytop, 1963; Davis, 1967; Gundogan, 2006). Tea is also considered commercially valuable in terms of secondary metabolites. Different cultivation methods affect the quality of tea by changing the biosynthesis of flavonoids. In low light conditions, the quality of tea drinks can be increased effectively by reducing the dose of flavonoids, which are the main compounds that contribute to leaf shrinkage (Wang *et al.*, 2011).
Tea plant is propagated in two ways as generative (seed) and vegetative (grafting, cutting and tissue culture). Generative technique; is carried out with the seeds formed as a result of the pollination of different tea types with each other (Ayfer *et al.*, 1987). Consequently, instead of a standard production, it leads to the formation of tea gardens with hybrid characters. Therefore, this production technique is not one of the preferred methods in today's modern tea agriculture. Although the vegetative propagation technique is an effective propagation method, tea and other related species (i) have slower reproduction

rates, (ii) some tea growing areas do not have suitable tea seedlings for planting due to dormancy and drought, (iii) the roots of some clones are lost because of various problems such as poor survival rate in the nursery due to its weak condition, (iv) the seasonal rooting ability of cuttings. Considering these problems, the micropropagation technique seems to be an ideal choice for solving the problems associated with traditional production. It is also suitable for the development of tea varieties that are in high demand in the industry and capable of rapid production, and can supply large quantities in a short time (Mondal, 2014).

Tissue culture methods allow production in a shorter time than traditional reproduction methods (Wachira and Ogada, 1995; Ponsamuel *et al.*, 1996; Bag *et al.*, 1997; Akula and Akula, 1999; Akula *et al.*, 2000; Mondal *et al.*, 2000). However, there are some factors that limit the micropropagation of the tea plant and prevent its commercialization. One of them is the problem of youth loss, which is frequently encountered in *in vitro* reproductions. It is also thought that micropropagated tea plants may be very sensitive to drought due to their weak root structures (Dodd, 1994). As an alternative to solving these problems, significant advances have been made in the *in vitro* propagation of tea varieties through direct or indirect organogenesis over the past 20-30 years (Sarathchandra *et al.*, 1997; Gunasekare and Evans, 2000a). The technique of *in vitro* propagation through organogenesis is a two-step process involving the growth of shoots and roots. In addition, somatic embryogenesis is a one-step procedure to create a bipolar structure and therefore this system saves labor, time, space and money in the production of plants (Dodd, 1994).

Elite tea production is carried out by clonal selection. However, it is possible to produce elite tea from the existing tea varieties. Improvement of tea depends on the availability of sufficient genetic information. Therefore, it is necessary to increase the genetic diversity of the tea species. Theoretically, indirect regeneration leads to plants with genetic diversity (Wu *et al.*, 1981; Frish and Camper, 1987; Gunasekare and Evans, 2000b). Therefore, somatic embryogenesis can be applied to generate somaclonal variants. Thus, *in vitro* screening will be possible to select the few desired cultivars. Moreover, somatic embryos with genotypes of selected elite parents are a potential resource for the production of artificial seeds, germplasm storage, genetic transformation as well as genetic and biochemical studies. The use of somatic embryogenesis in tissue culture applications has wide biotechnological applications due to its potential to bring widespread changes (Lutz *et al.*, 1986; Tulecke, 1987).

A high auxin/cytokinin ratio is required for somatic embryogenesis in the tea plant (Vieitez, 1990). It has been determined by studies that exogenous cytokinin generally suppresses embryo development, but cytokinins applied *in vitro* conditions in tea plant promote somatic embryo induction and development from zygotic embryo and cotyledon explants (Ammirato, 1983; Das and Barman, 1988; Nakamura, 1988).

In the current study, it is aimed to establish the appropriate procedure by examining different parameters such as determining the carbon sources, suitable plant growth regulator and dose, which are effective on embryonic callus regeneration obtained from the leaves of the tea (*Camellia sinensis* (L.) O. Kuntze) plant, as well as examining different parameters such as the effect of culture conditions.

MATERIALS AND METHODS

Material

In the study, leaf explants taken from *in vitro* plantlets of the domestic "Ali Rıza Erten" tea variety bred by Atatürk Horticultural Research Institute and clonally micropropagated at a commercial company was obtained were used. The leaves of these *in vitro* plantlets, which are being reproduced in Ege University, Engineering Faculty Bioengineering Department, Plant Cell, Tissue and Organ Culture Laboratory, were used as starting material. The lengths of these explants were determined to be 1-1 cm.

Method

Preparation of Nutrient Media for Culture of Explants: All nutrient media used in the study were prepared based on semi-solid MS (Murashige and Skoog 1962). In all experiments, 3% sucrose was used as the carbon source (except for the carbon source trials) and also 0.3% gelrite as the gelling agent and the pH

was adjusted to 5.8. 210 mL jars were used as the culture container and 25 ml of nutrient media was added to each jar. Sterilization of the nutrient media was carried out by autoclaving. This process, which continues for 15 minutes, takes place at 121ºC and 1 atm pressure. After the autoclave process, sterile nutrient media were taken into the cabinet and stored properly until used.

The content of the nutrient media to be prepared to determine the effect of plant growth regulator at different doses on the embryonic callus regeneration in the tea plant, nutrient media to be prepared to determine the effect of different carbon sources at increasing doses, and finally nutrient media prepared for the culture conditions experiments are given in Table 1. Experiments were carried out in 3 replications. Minitab 17 Statistical Software (Minitab Inc, PA, USA) program was used to evaluate the obtained data.

Table 1: Different carbon sources and doses, different growth regulators and doses, and nutrient media compositions created with culture conditions

Trial	Media code	Media composition
Different carbon sources and different doses experiment	ZKM1	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+10 g L^{-1} maltose
	ZKM2	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+20 g L^{-1} maltose
	ZKM3	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+30 g L^{-1} maltose
	ZKM4	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+40 g L^{-1} maltose
	ZKG1	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+10 g L^{-1} glucose
	ZKG2	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+20 g L^{-1} glucose
	ZKG3	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+30 g L^{-1} glucose
	ZKG4	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+40 g L^{-1} glucose
	ZKS1	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+10 g L^{-1} sucrose
	ZKS2	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+20 g L^{-1} sucrose
	ZKS3	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+30 g L^{-1} sucrose
	ZKS4	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA+40 g L^{-1} sucrose
Trial of IAA at different doses	ZO0	MS+4 mg L^{-1} BAP+0 mg L^{-1} IAA
	ZO1	MS+4 mg L^{-1} BAP+1 mg L^{-1} IAA
	ZO2	MS+4 mg L^{-1} BAP+2 mg L^{-1} IAA
	ZO4	MS+4 mg L^{-1} BAP+4 mg L^{-1} IAA
Trial of KIN at different doses	ZS0	MS+1.5 mg L^{-1} IBA+ 0 mg L^{-1} KIN
	ZS1	MS+1.5 mg L^{-1} IBA+ 1 mg L^{-1} KIN
	ZS2	MS+1.5 mg L^{-1} IBA+2 mg L^{-1} KIN
	ZS4	MS+1.5 mg L^{-1} IBA+4 mg L^{-1} KIN
Culture condition trial	ZKA	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA (photoperiod:16 h light/8 h dark)
	ZKK	MS+4 mg L^{-1} BAP+1.5 mg L^{-1} IBA (dark)

RESULTS AND DISCUSSION

Determination of the effects of different carbon sources and doses on globular embryo regeneration in tea plant

Each trial was established by culturing 30 explants. After the explants were cultured, weekly observations were made and the data obtained at the end of the 40-days culture period were shown in Table 2, Fig. 1 and 2.

Table 2: Effects of different carbon sources and doses on embryogenic callus regeneration percentages in *Camellia sinensis* (L.) O. Kuntze (±SE) [1]

Doses of carbon source		10 mg L^{-1}	20 mg L^{-1}	30 mg L^{-1}	40 mg L^{-1}	AVG
Carbon source	Maltose	60.00±5.77 ab	16.67±3.33 cd	16.67±3.33 cd	3.33±3.33 d	24.17 b
	Sucrose	43.33±6.67 bc	80.00±10.0 a	76.67±8.82 a	53.33±8.52 ab	63.33 a
	Glucose	53.33±3.33 ab	10.00±0.00 d	6.67±3.33 d	0.00±0.00 d	17.50 b
AVG		52.22 a	35.55 b	33.33 b	18.89 c	

[1]Applications were performed in triplicate and 10 explants were used for each replication. The differences between the values indicated with different letters are significant at the p<0.0.1 level according to the Duncan multiple comparison test. SE: Standard error

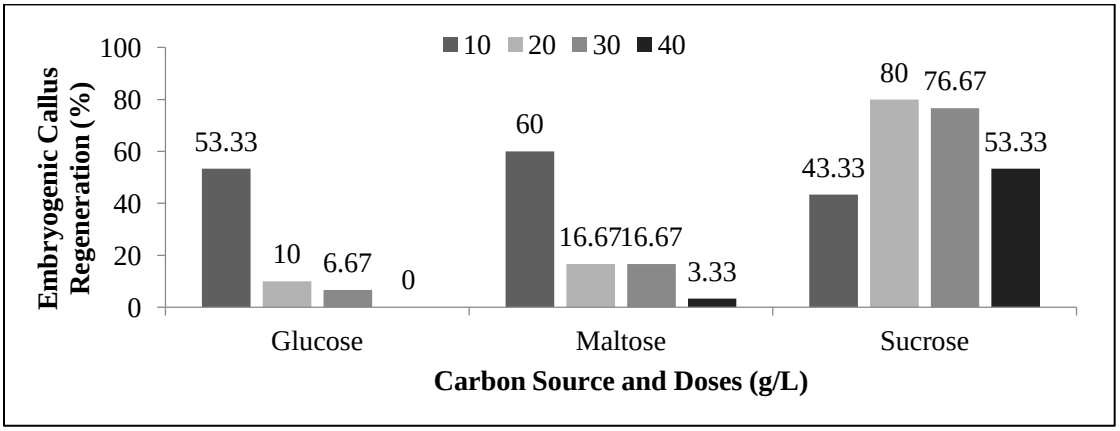

Figure 1: The effect of different carbon sources and doses on embryogenic callus regeneration (%) in *Camellia sinensis* (L.) O. Kuntze

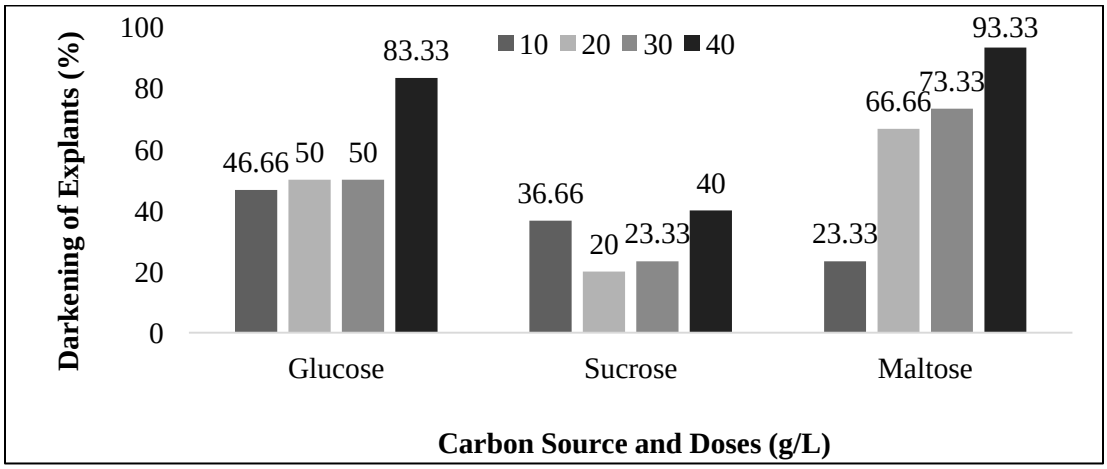

Figure 2: Effect of different carbon sources and doses on darkening of explants (%) in *Camellia sinensis* (L.) O. Kuntze species

Callus regeneration began to be observed on the 20th day in the cultured explants and all calli had embryogenic character (Tab. 2). The obtained calli which is green-cream colored has a hard structure as physiological state (Fig. 3). Statistically, carbon source, dose and interactions were found to be

significantly different. When carbon sources were evaluated among themselves, it was determined that sucrose was more effective in globular embryo regeneration than other carbon sources. When the doses were compared, 10 mg L^{-1} (43.33%) was determined as an effective dose and it was determined that increasing doses reduced regeneration. Examining the carbon source dose interaction, 20 (80%) and 30 mg L^{-1} (76.67%) sucrose gave the highest globular embryo regeneration. The relationship between the three media compositions determined to minimize callus regeneration is seen as follows: ZKG4>ZKM4>ZKG3. When the data were evaluated, it was observed that the increase in glucose dose inhibited callus formation and promoted darkening. As stated in the literature, high dose of sugar has been observed that inhibits the expression of genes involved in photosynthesis and has a growth inhibitory effect (Reekie and Bazzaz, 1987; Rolland *et al.,* 2006; Yaseen *et al.,* 2013).

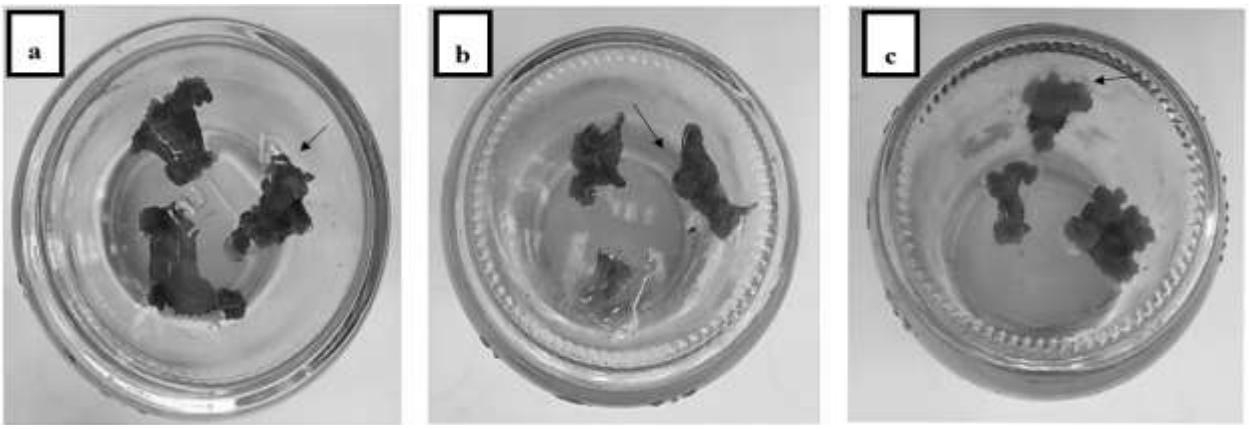

Figure 3: Applications for embryonic callus regeneration in different models in the same applications: *a. ZKM1, b. ZKG1, c. ZKS1 (Embryos in globular stage are shown with arrow)*

When the darkening explant rate is examined, it was observed that the highest rate was observed in ZKM4 (93.33%) nutrient media. The other three nutrient media with the highest darkening rate are listed as follows: ZKG4 >ZKM3 >ZKM2. The carbon source, which has a lower percentage of darkening compared to other carbon sources, is sucrose.

Different carbon sources show quite different effects on plant explants in terms of morphogenetic properties. Therefore, in order to evaluate the effects of different types and doses of carbon sources, our observation period has been adjusted to be weekly and our culture period to be 40 days. Sugars are the most important and complex components of the nutrient medium. *In vitro* plant cells and tissues are not fully autotrophic. These heterotrophic expanders require carbohydrates in the nutrient medium to maintain osmotic potential and to provide sufficient energy for processes that use high energy such as embryogenesis and organogenesis (Jain and Babbar, 2003). In addition to acting as a carbon source, sugars are inducers of desiccation tolerance by providing an osmotic environment during maturation and germination of somatic embryos (Mondal *et al.,* 2000a). Sucrose is the most used sugar in the media for plant tissue culture. The reason is that, it is located in the stem phloem of the plant (Thomson and Thorpe, 1987). The morphogenic pathway is also affected by the type of carbon source added during callus formation and plant regeneration. When sucrose was replaced by maltose in wheat anther cultures, androgenic embryos instead of callus were formed in both relatively highly androgenic responsive genotypes and recalcitrant genotypes (Orshinsky *et al.,* 1990). In barley anther cultures, maltose and malt extract supported the embryogenic pathway compared to sucrose (Finnie *et al.,* 1989). Overall, maltose appears to be superior to sucrose for green plant regeneration from anther and isolated microspore culture systems of grass species (Petersen et. al., 1999).

High concentrations of sugar can have a growth inhibitory effect. Regarding this, Yıldız et al. (2007) on three sugar beet lines, it was noted that at higher sucrose concentrations in the culture medium, growth

inhibition in shoot regeneration, phenolic secretion and tissue necrosis increased. While it has been stated that high levels of carbohydrates can inhibit the expression of genes involved in photosynthesis, it has been determined that they increase the expression of genes involved in nitrate assimilation, growth, storage and starch remobilization (Reekie and Bazzaz, 1987; Rolland *et al.*, 2006; Yaseen *et al.*, 2012).

Determination of the effects of different doses of IAA and KIN growth regulators on globular embryo regeneration in Camellia sinensis (L.) O. Kuntze

The expression "phase cycle", which is frequently used for development and cell cycle in plants, is also used to express the changes between dedifferentiation-redifferentiation stages of explants in tissue culture and micropropagation. Phase change in tissue culture is affected by many factors, especially plant growth regulators. Auxins and cytokinins, which are important plant growth regulators, play critical roles in DNA duplication, nucleic acid metabolism, synthesis of various proteins, and mitosis and cytokinesis. In addition to being important elements in plant tissue culture, auxin and cytokinins are also used for callus initiation and plantlet regeneration (Gao *et al.*, 2019).

Auxin-type plant growth regulators are the oldest hormones used in agriculture (Halloran and Kasım, 2002). These are substances that cause cell enlargement and growth, and promote events such as cell elongation, tissue development and root formation. When used alone in tissue cultures, auxin hormone provides callus stimulation and rooting of the resulting shoots (Seçer, 1989).

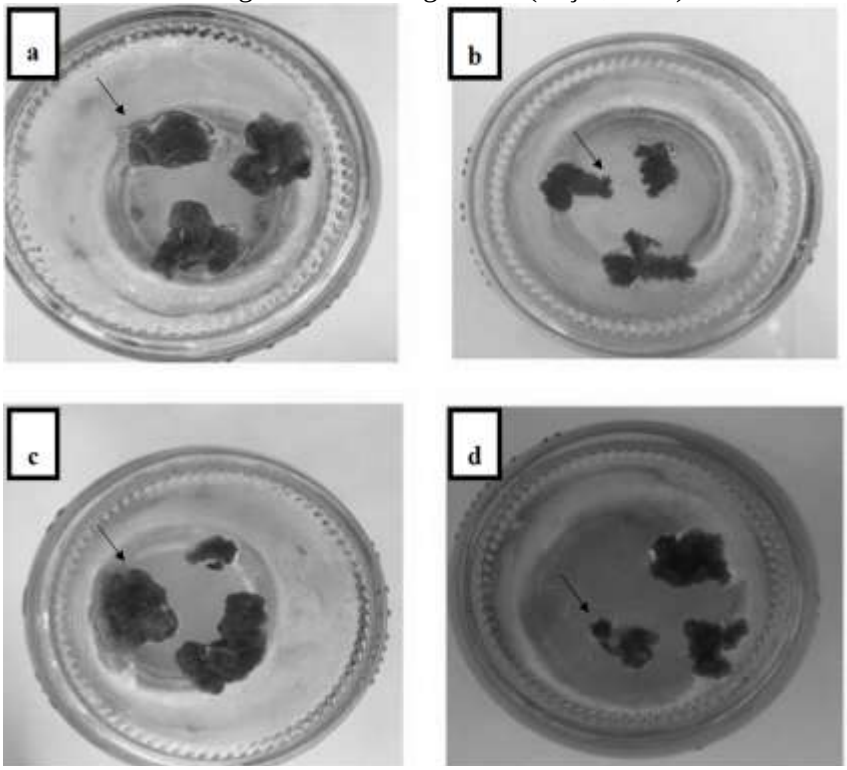

Figure 4: Effects of plant growth regulators (IAA and KIN) at different doses on callus physiology: *a. ZS2(MS+1.5 mg L^{-1} IBA+2 mg L^{-1} KIN), b.ZS4 (MS+1.5 mg L^{-1} IBA+4 mg L^{-1} KIN), c.ZO2 (MS+4 mg L^{-1} BAP+2 mg L^{-1} IAA), d.ZO4 (MS+4 mg L^{-1} BAP+4mg L^{-1} IAA) (Embryos in globular state are shown with arrow)*

Kinetin, benzyl adenine and zeatin are widely used in plants. Although generally found in young tissues, many kinetin-like substances are isolated from germinating seeds, sap and young fruits. It is especially synthesized in root meristems and then transported to the green tissues of the plant via xylem. They are known as hormones that are effective in cell division and delay aging. In addition, while auxins promote root formation, cytokinins promote shoot formation. They generally provide organ formation and

development in tissue culture media. Depending on the balance of these two compounds, root and shoot formation can be controlled. Although the application of IAA+KIN causes rapid cell division and the cells to remain in a continuous meristematic state, unorganized new cells continue to form in the callus tissue in the case of roughly equal concentrations of auxin and stokinin (Güleryüz, 1982; Westwood, 1993; Kaynak and Ersoy, 1997).

Callus initiation was observed on the 18th day in culture. When the physiological state of the obtained calli was examined, it was determined that they had a green color and a hard structure (Fig. 4). In plant growth regulators added to the nutrient media, auxins were that most affected the plant growth regulators for somatic embryo regeneration. It has been reported in the literature that when used alone in tissue cultures, auxin provides callus stimulation and rooting of the shoots (Bidlack and Jansky, 2011). On the other hand, although auxin is used to stimulate the somatic embryo, the constant presence of auxin in the media prevents the development of somatic embryos (Parrott *et al.*, 1993). According to the statistical analysis; while plant growth regulator type is not seen as an effective factor, the interaction of dose and plant growth regulator is statistically significant (Tab. 3). It was determined that increasing plant growth regulator doses increased embryogenic callus regeneration and the best results were obtained in ZO2, ZO4, ZS1, ZS2 and ZS4 nutrient media (Fig. 5).

Table 3: Embryogenic callus regeneration percentages (±SE) of plant growth regulators on *Camellia sinensis* (L.) O. Kuntze

Plant growth regulator type	Plant growth regulator dose (mg L^{-1})	Callus regeneration percent ±SE	AVG
IAA	0	13.33±3.33 c	60,00
	1	50.00±0.00 b	
	2	83.33±3.33 a	
	4	93.33±3.33 a	
KIN	0	13.33±3.33 c	64,17
	1	76.67±8.82 a	
	2	93.33±3.33 a	
	4	73.33±3.33 a	

**Applications were performed in triplicate and 10 explants were used for each replication. The differences between the values indicated with different letters are significant at the p<0.0.1 level according to the Duncan multiple comparison test. SE: Standard error*

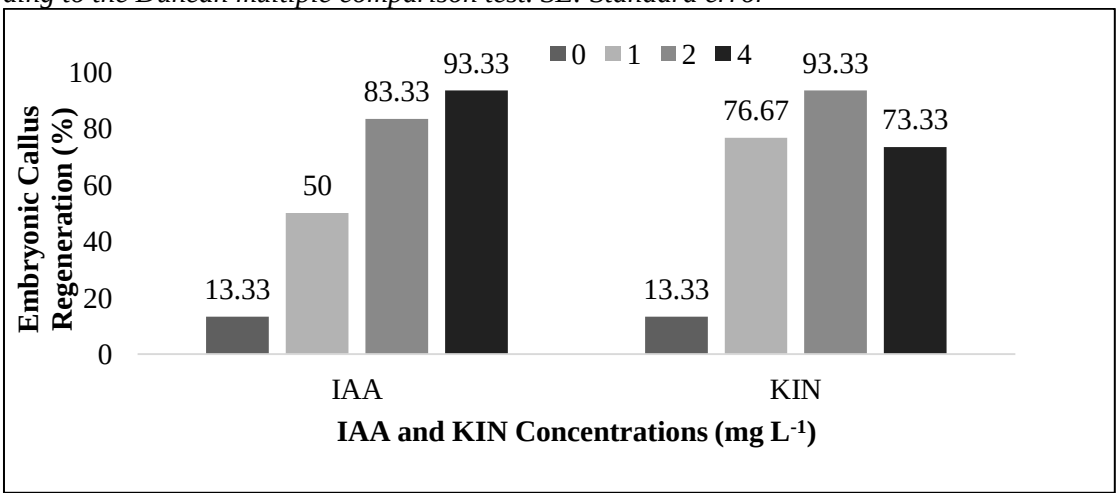

Figure 5: Effect of plant growth regulators (IAA and KIN) on embryogenic callus regeneration (%) in tea plant

In the studies carried out by Mondal (2000b) with different tea plant genotypes, somatic embryogenesis regeneration has been observed 11.1% for TG207/2/B variety and 50% for UPASI-9 variety in MS nutrient media containing 2.5 mg L^{-1} NAA and 0.2 mg L^{-1} BAP. In the Ali Rıza Erten tea variety used in this study, 93.33% embryogenic callus regeneration was observed. Although genotype is an effective factor, it can be said that embryogenic callus regeneration occurs at a higher rate as a result of the study. In addition, it was concluded that high embryogenic callus regeneration rate (93.33%) was obtained in nutrient media containing high auxin and cytokinin (4 mg L^{-1} IAA + 4 mg L^{-1} BAP), increasing plant growth regulator doses increased somatic embryogenesis.

Kato (1996) and Sarathchandra et al. (1988) used leaves as starting explants in indirect somatic embryogenesis. Kato (1996) cultured different concentrations of 2,4-D into MS medium and had considerable success in inducing somatic embryos directly or indirectly from leaf explants. Also, in NAA applications, it was more effective than 2,4-D when used alone or in combination with BAP for induction of embryogenic callus and somatic embryos from seeds of *Eschscholzia californica* (Park and Facchini, 1999).

A high auxin/cytokinin ratio is required for somatic embryogenesis in *C. sinensis* (Vieitez 1990). The selection of auxins used in these studies was limited to 2,4-D, IAA and IBA. Exogenous cytokinin generally suppresses embryo development, but cytokinins applied to *C. sinensis* culture promote somatic embryo induction and development from zygotic embryo and cotyledon explants (Ammirato, 1983, Das and Barman, 1988, Nakamura, 1988).

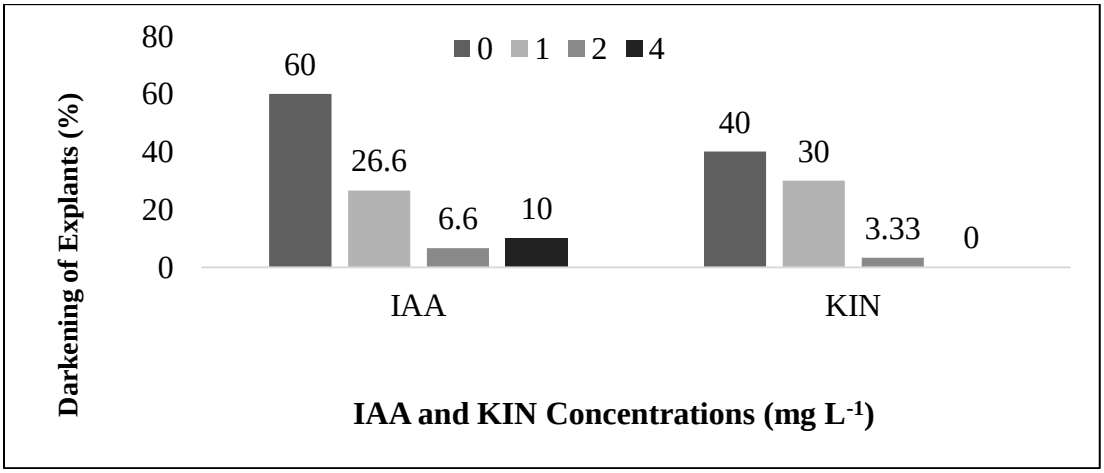

Figure 6: Effect of plant growth regulators (IAA and KIN) on tea plant darkening of explants (%)

When we examined the darkening percentages, it was observed that the media with the highest darkening percentage (60%) was ZO0 (Fig. 6). There was no darkening observed in explants cultured on ZS3 medium (Table 4). Darkening seen *in vitro* is a chemically explained phenomenon caused by oxidized polyphenols and tannins. Various applications are made to prevent this phenomenon in explants. One is to add a plant growth regulator and another is to reduce ambient light. In extensive studies, different combinations of plant growth regulators were investigated and it was revealed that low auxin/cytokinin ratio or administration of low doses of cytokinins promoted somatic embryo regeneration. BAP has been found to be effective in somatic embryo regeneration in *Camellia japonica*, *C. sasanqua*, *C. chrysanthaand* and *C. reticulata*, as well as the plant growth regulator tea plant, but it has been reported that KIN can also be used (Mondal *et al.*, 2004). Wachira and Ogadaa (1994) showed in their study that callus formation increased from 53% to 60% with the reduction of BAP+KIN doses in the nutrient media. In our study, it was concluded that increasing plant growth regulator doses increased embryogenic callus regeneration, contrary to the studies.

Table 4: Effect of plant growth regulators (IAA and KIN) on tea plant darkening of explant (%)

	Darkening of explant (%)			
Doses (mg L^{-1})	0	1	2	4
IAA	60	26.6	6.6	10
KIN	40	30	3.33	0

Determination of the effect of culture conditions on globular embryo regeneration in Camellia sinensis (L.) O. Kuntze species

Callus initiation was observed on the 20th day in culture. While the physiological state of the calli obtained in the photoperiod trials has a green color and a hard structure, the structure of the calli in the dark environment trials is cream-pink colored and soft. Based on the data obtained as a result of the analysis, it was determined that the photoperiod was effective on callus regeneration (Fig. 7). Considering the percentage of darkening explants, which is another examination parameter, it was observed that the number of darkening explants was higher in dark environment trials (Tab. 5).

Table 5: Effects of culture conditions on embryogenic callus regeneration percentages on *Camellia sinensis* (L.) O. Kuntze

Culture conditions	Percentage of callus regeneration (%)
ZKA	83.33
ZKK	53.3

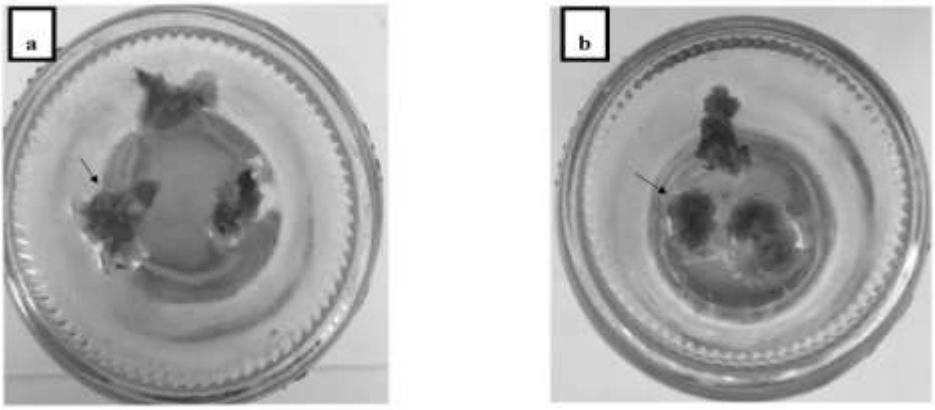

Figure 7: Effect of culture conditions on callus physiology: *a.ZKK (dark), b.ZKA (photoperiod: 16 hours light/8 hours dark) (Embryos in globular state are shown with arrow)*

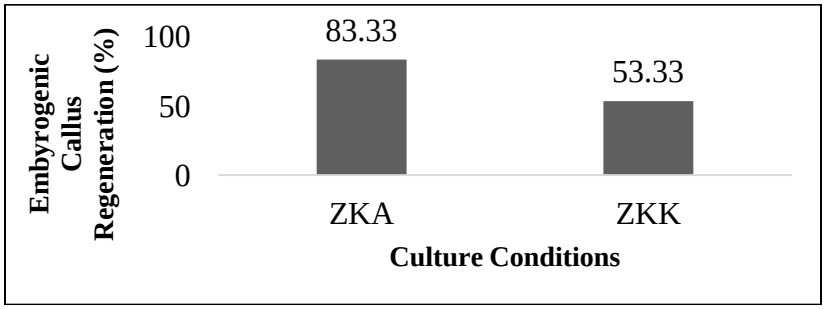

Figure 8: Effect of culture conditions on embryogenic callus regeneration (%) in *Camellia sinensis* (L.) O. Kuntze *(ZKA:photoperiod: 16 hours light/8 hours dark, ZKK: dark)*

The tea plant is a very rich plant in terms of secondary metabolites. It has also been stated by many researchers that light is an environmental parameter that has a significant effect on the mechanisms of these secondary metabolites. Tea (*Camellia sinensis* (L.) O. Kuntze) is also considered commercially valuable in terms of secondary metabolites. Different cultivation methods affect the quality of tea by changing the biosynthesis of flavonoids. In low light conditions, the quality of tea drinks can be increased effectively by reducing the concentration of flavonoids, which are the main compounds that contribute to leaf shrinkage. The aim of the study by Wang et al. (2011) was to analyze the effect of low-light environment on flavonoid biosynthesis in relation to the expression of flavonoid pathway genes in tea leaves. The data of the study revealed that low-light environment had significant effects on both flavonoid and lignin biosynthesis, but not on anthocyanin accumulation. Among all detected compounds, the concentration of PA and *O*-glycosylated flavonols in leaves grown in low-light conditions decreased by 53.37% and 43.26%, compared to leaves exposed to sunlight, and changed more than other compounds. More research is needed to understand the relationship between phenolic acids and other flavonoid compounds in tea plants (Wang *et al.,* 2011). It has been reported that dark culture conditions have a negative effect on providing direct or indirect somatic embryo regeneration, especially from root and leaf explants of the tea plant (Mondal *et al.,* 2004). As a result of our study on trials with the same nutrient media compositions; photoperiod has been found to have a positive effect on callus regeneration (Fig. 8).

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