TITLE: The study of morphogenesis in tissue culture of elite cotton varieties of Kazakhstan

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ABBREVIATIONS: MS – Murashige and Skoog medium; 2,4-D – 2,4-Dichlorphenoxyacetic acid; EAA – ethanol-acetic acid; CH – casein hydrolisate; NAA – Naphthylacetic acid; PCD – programmed cell death; ECC – embryogenic cell complexes

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ABSTRACT

Revelation of common cytophysiological regularities of morphogenesis in vitro for various genotypes is the productive approach used in our laboratory for the elaboration of genotype-independent protocols of long-term regeneration in vitro for "recalcitrant" agricultural crops. The purpose of given research is the study of morphological heterogeneity of callus tissues of elite cotton varieties of Kazakhstan and revealing the peculiarities of their metamorphosis and hystological structure.

Three types of primary callus tissues have been identified in primary culture of hypocotyls and cotyledons of seven elite cotton (*Gossypium hirsutum* L.) varieties: I – grayish-white friable; II – white-dim dense; III – dark-brown. Grayish-white morphogenic globular calli (IV type) and three types of embryogenic calli has been obtained during subculture of a primary tissues: friable translucent yellowish (V type), friable dim white (VI type) and compact calli (VII type).

Embryogenic suspension cultures have been obtained from two types of friable embryogenic calli of two elite cotton varieties: Maktaaral 4005 (VI type) and Maktaaral 4006 (V type).

Grayish-white friable callus (I type) has been identified as a common for most of tested genotypes and both types of explants and selected as a morphogenically perspective for the obtaining of embryogenic tissues. It was shown that callus types differed each from other on their hystological structure. At the same time, all types of embryogenic calli have, on the whole, the similar structure and differ each other from on the size of embryogenic complexes, stages of embryoids development and proportion of cells with signs of programmed cell death.

Key Words:

Cotton (*Gossypium hirsutum* L.), callus tissues, morphogenesis in vitro, hyistological structure, somatic embryogenesis, cell suspension culture

Cotton *(Gossypium hirsutum L.)* is one of the major agricultural crops that has an export importance in Kazakhstan. Besides, cotton is a basis for the development of an own cotton-textile industry being one of the seven major clusters of the economic development in Kazakhstan. In this connection there is rather relevant question on the creation of elite cotton cultivars with high quality production, adapted to local soil-climatic and phyto-sanitarian conditions.

Genetic engineering is one of the most effective and modern ways to the direct purposeful changing of plant genotype. Development and wide use of genetic transformation technology for cotton improvement is restrained by the unsolvency of a problem of regeneration in vitro for this crop – by the essential dependence of in vitro morphogenesis processes from an initial genotype and by the loss of the ability for plant regeneration during a long-term subculture (Trolinder and Xhixian, 1988b; Smith and Park, 2004). This fact, in turn, considerably constrains the creation of effective recipient systems for cotton genetic transformation. As a result, transgenic plants with valuable attributes created in the world are based on high-regenerable genotypes, which usually are not commercially important (Smith and Park, 2004). Therefore, one of the major research problems in the field of cotton biotechnology is the development of long-term plant regeneration technologies which would be applicable to wide spectrum of cotton elite genotypes.

The strategy of our research focuses on revealing cytophysiological regularities of morphogenesis *in vitro* common for various genotypes and on this basis - on elaboration of genotype-independent long-term regeneration technologies of important agricultural crops (wheat, barley, cotton) for the use in genetic transformation.

From our perspective the listed difficulties in cotton regeneration, as well as in many other recalcitrant crops (wheat, barley, soybean), are caused by lack of knowledge about

common cytophysiological regularities of morphogenesis in vitro for various genotypes. Our strategy on the development of genotype-independent regeneration technologies of wheat and barley based in detailed investigation of morphological heterogeneity and metamorphosis of calli, hystological characteristic of various morphotypes and in finding of common morphogenically perspective calli types for various genotypes from which long-term regenerated tissues could be initiated (Rakhimbayev et al., 1992; Denebayeva and Bishimbayeva, 2000; Bishimbayeva, 2006a). Common cytophysiological features of the regulation of somatic embryogenesis and regeneration in vitro have been revealed in our laboratory for various genotypes of wheat and barley (Bishimbayeva and Denebayeva, 2001; Amirova and Bishimbayeva, 2004). As a result, we have developed genotype - independent technology of long-term regeneration (10-12 subcultures and more) from callus and suspension cultures of anyone earlier "recalcitrant" commercially important varieties and lines of wheat and barley (Bishimbayeva et al., 2003).

We suppose the use of same strategy in the cotton biotechnology in general could allow researchers to pass from the empiric approach to purposeful regulation of morphogenesis *in vitro* and to overcome a problem of genotype dependence in cotton long-term regeneration *in vitro*.

The given research represents the first stage in the realization of the strategy that was above mentioned. The purpose of present investigation is the study of morphological heterogeneity of callus tissues of the elite cotton varieties of Kazakhstan, revelation the peculiarities of their metamorphosis and histological structure.

MATERIALS AND METHODS

Commercially important elite cotton *(Gossypium Hirsutum* L.*)* varieties of Kazakhstan - Maktaaral-4003, Maktaaral-4005, Maktaaral-4006, Maktaaral-4007, Maktaaral-4011, Maktaaral-4019, Pakhtaaral -3044, have been used as objects of investigation.

Hypocotyls (0,4-0,5 см) and cotyledons (1 sm^2) isolated from 5-7 day time seedlings of seven cotton varieties were placed on the solid MS medium (Murashige and Skoog, 1962) with 1,0 mg/l 2,4-D or 0,1 mg/l 2,4-D and 0,5 mg/l kinetin (Trolinder and Goodin, 1988a). Primarily induced callus tissues were subcultured according the protocol of Kumria et al. (2003), or of Ul-Haq (2005), or under the protocol developed in our laboratory for tissue culture of wheat and barley (Bishimbayeva et al., 2003). Morphology of callus tissues have been determined visually and by the use of stereoscopic microscope MBE 10C (Russia).

Suspension cultures have been initiated from embryogenic callus tissues: the ratio of callus weight and volume of nutrient media was equal to 2 g per 30 ml. Suspension culture was grown on the shaker with 120 rpm/min. Seeds, calli and suspension cultures were incubated in the light-room with 16-hour photoperiod and temperature 26+2^oC.

Callus tissues with different morphological types were fixed for hystological study in Chemberlen fixator (Pausheva, 1988). Staining of paraffin sections was carried out by Shiff reagent, hematoxiline and alcian blue in series (Kamelina and Jinkina, 1992). For the obtaining of temporary preparations callus tissues were fixed in the ethanol-acetic acid (EAA) (3:1) with subsequent staining with 5 % acetocarmine. Living calli were stained by methylene blue without fixation for the estimation of cells viability (Pausheva, 1988). Paraffin sections and temporary preparations were studied by the use of light microscope "Leika".

RESULTS AND DISCUSSION

According the most of protocols on the cotton genetic transformation (Sunilkumar and Rathore, 2001; Mishra et al., 2003) hypocotyls and cotyledons isolated from 5-7 day seedlings have been used as a recipient systems for foreign genes. However, it was shown in the recent publications (Leelavathi et al., 2004) that highest amount of transformants was reached when embryogenic callus cultures directly exposed to transformation. Therefore, the basic attention in our research was given to the obtaining of embryogenic callus and cell cotton cultures.

Morphological heterogeneity and metamorphosis of callus tissues have been investigated. Three types of primary callus tissues induced from hypocotyls and cotyledons of seven domestic cotton varieties have been identified: I – grayish-white friable; II – white-dim dense; III – dark-brown (Fig. 1 a,b,c). Morphology of primary tissues differed in the accordance with genotype and hormonal status of medium (Table 1).

Grayish-white morphogenic globular calli (IV type) and two types of friable embryogenic calli has been obtained during subculture as a result of metamorphosis: translucent yellowish (V type) and dim white (VI type) (Fig.1 d,e,f). Also compact embryogenic calli (VII type) has been obtained (Fig.1 g).

It was revealed, that optimal conditions for the obtaining of globular morphogenic calli (IV type) are the initiation of grayish-white friable callus (I type) on the MS media with 0,1 mg/l 2,4-D and 0,5 mg/l kinetin and subsequent subcultivation during the two subculture on the MS media with 1,0 mg/l 2,4-D, 1000 mg/l casein hydrolisate (CH), 500 mg/l proline, 3% maltose (Bishimbayeva et al., 2003).

Friable embryogenic calli of Maktaaral-4005 (type VI) and Maktaaral-4006 (type V) has been initiated through the obtaining of grayish-white friable callus (I type) on the MS media with

0,1 mg/l 2,4-D, 0,5 mg/l kinetin (Trolinder and Goodin, 1988) and 3% sucrose and its subsequent subcultivation during the two subcultures according the protocol of Ul-Haq (2005): a) MS media + 2mg/l NAA (Naphthylacetic acid) + 0,1 mg/l kinetin, 0,1 mg/l zeatin; b) MS media with high concentration of NH_4NO_3 (+3,55 g/l) MS media with double concentration of KNO_3 (3,8 g/l).

Compact green embryogenic calli (VII type) have been produced as a result of two subculture of IV tissue type according the protocol of Kumria et al. (2003): a) MS media with 3,8 g/l KNO_3 and 3% maltose; b) 1/5 mineral salts of MS medium with 1% maltose.

All types of morphogenic tissues have been initiated from both types of explants – hypocotyls and cotyledons. Grayish-white friable callus (I type) has been identified as a common for most of tested genotypes and both types of explants and selected as a morphogenically perspective for the obtaining embryogenic tissues. Long-term embryogenic callus tissues have been produced from two elite cotton varieties - Maktaaral 4005 (type VI) and Maktaaral 4006 (V type).

Hystological Study of Callus Types

We have studied the hystological structure of different callus types. It was revealed that grayish-white friable callus (I type) consists from parenchyma cells, closely connected with each other, separately situated elongated callus cells and small cells with spherical shape (Fig. 2). Earlier we have shown that the presence of parenchyma cells, closely connected with each other, is the sign of morphogenetic perspectivity of intermediate callus tissues (Rakhimbayev et al., 1992). Appearance of separated small cells with spheric shape is one of the signs of embryogenic

competence (I.K. Vasil, 1985). Thus, hystological structure of type I callus demonstrates its potential to produce morphogenic and embryogenic tissues.

On the whole, structure of IV type of calli is similar with type I, but it differs from type I by the presence of densely packed meristematic cells among the parenchyma cells, the appearance of cells with signs of programmed cell death (PCD) and large amount of tracheal elements (Fig. 3 a,b). Signs of PCD have been identified by the staining of cells by methylene blue and acetocarmine: cytoplasm was shrunken, periplasmatic space was appeared, cell walls were thickened and nuclear material was released into the cytoplasm (McCabe et al., 1997; Bishimbayeva, 2006 b).

It was revealed, that all types of embryogenic calli (V, VI, VII) have the same structure with each other and contain embryogenic cell complexes (ECC), which consist of densely packed parenchima cells and are characterized by the high ratio of cells with signs of PCD (photo is not presented). ECCs of embryogenic calli constantly produce competent cells and embryoids in different stages of differentiation – two-, three-, four- celled proembryos and preglobular stages. Types of embryogenic calli differ each from other on the size of ECCs, stages of embryoids, ratio of cells with signs of PCD, competent cells and embryoids.

Obtaining of Embryogenic Suspension Cultures

Friable embryogenic calli of type V (Maktaaral 4006) and type VI (Maktaaral 4005) have been inoculated in the liquid nutrient MS media with 0,1 mg/l 2,4-D. Embryogenic suspension cultures have been established after 20 days of culture.

Cytological study showed that suspension cultures consisted from competent cells, two-, three-, four- celled proembryos and embryoids on preglobular stages (Fig. 4 a, b). Cells with signs of PCD have been identified in the obtained cell cultures (Fig. 4 a, b).

Conclusions

Obtained data gave us the promising results confirming the physiological strategy which has been proposed by I. Vasil (1985) and successfully used earlier in our laboratory as a main approach in the elaboration of cell technologies for cereals (Rakhimbayev et al., 1992; Bishimbayeva, 2006a). Our investigation showed that type I callus is appeared to be a common for most tested cotton genotypes. It was revealed that different types of morphogenic and embryogenic callus tissues could be induced from type I callus. Moreover, all types of embryogenic calli have, on the whole, similar structure and differ each from other on the sizes of ECCs, stages of embryoids and proportion of cells with signs of PCD.

Obtaining of embryogenic tissues and cell cultures of two elite Kazakh cotton varieties – Maktaaral 4005 and Maktaaral 4006, is a practical result of cytomorphological study. Preliminary results presented in this paper allow us to expect the finding of tissue types common for wide spectrum of cotton genotypes, the revealing of the regularities of in vitro morphogenesis in these “universal” tissues and the elaboration on this basis genotype independent cell and transformation technologies for the earlier “recalcitrant” elite cotton varieties.

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Table 1– Morphological heterogeneity of of primary callus tissues of various cotton cultivars

	Cultivars	Callus types, %					
		MS media + 0,5 mg/l kinetin + 0,1 mg/l 2,4-D			MS media + 1,0 mg/l 2,4-D		
		I	II	III	I	II	III
1	M-4003	37,5	37,5	25,0	40,0	-	60,0
2	M-4005	55,0	11,7	33,0	55,0	11,0	34,0
3	M-4006	18,0	9,2	63,6	12,5	-	87,5
4	M-4007	30,0	25,0	45,0	7,2	14,3	78,5
5	M-4011	14,3	28,6	57,1	-	-	100,0
6	M-4019	-	66,6	33,4	-	40,0	60,0
7	П-3044	-	66,6	33,4	50,0	-	50,0

Symbols: I – grayish-white friable; II – white-dim dense; III – dark-brown

FIGURE CAPTIONS

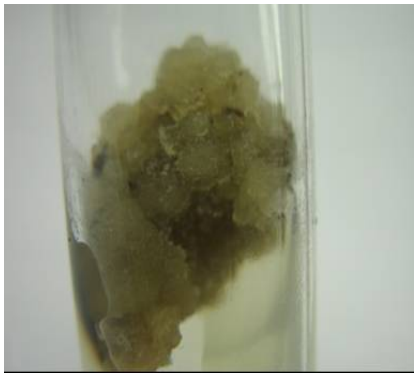
Figure 1. Morphological types of cotton callus tissues.

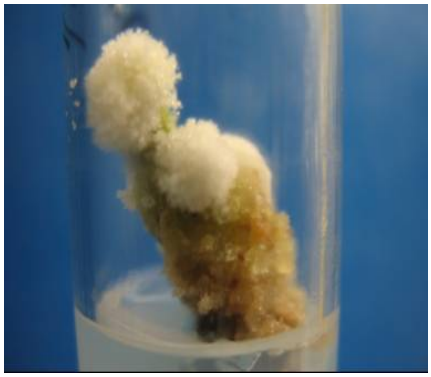
Symbols: a) – grayish-white friable (I type), b) – white-dim dense (II type), c) – dark-brown (III type), d) – grayish-white morphogenic globular calli (IV type), e) – friable embryogenic translucent yellowish (V type), f) – friable embryogenic dim white (VI type), g) – compact embryogenic (VII type).

Figure 2. – Hystological structure of grayish-white friable callus (I type) (stained in series by Shiff reagent, hematoxyline and alcian blue). (⟶) – separated elongated callus cells, (⋯⋯▸) - small cells with spherical shape.

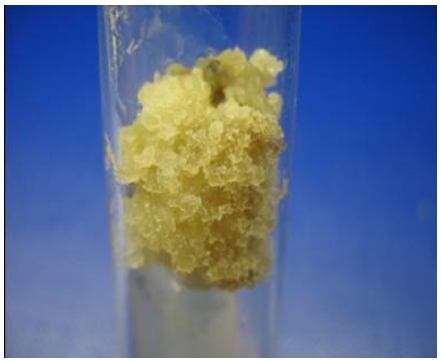
Figure 3. – Hystological structure of grayish-white morphogenic globular calli (IV type) (stained in series by Shiff reagent, hematoxiline and alcian blue). **a** - meristematic cells among the parenchyma cells. **b** - appearance of large amount of tracheal elements and cells with signs of PCD. Symbols**:** (⟶) – meristematic cells; (⋯⋯▸) – parenchima cells; (●) – cell with signs of PCD; (– · – ·▸) - tracheal elements.

Figure 4. - Cells and embryogenic structures of cotton suspension culture (stained by methylene blue). **a** - fragment with initiated 2-3 celled proembryos. **b** – fragment with preglobular stage embryo. Symbols: (⋯⋯▸) - competent cells; (⟶) – cells on the stages of initiation (2-, 3-, 4-celled proembryos); (●) – cell with signs of PCD; (– · – ·▸) – preglobular stage embryo;





a **b** **c**



d **e** **f**

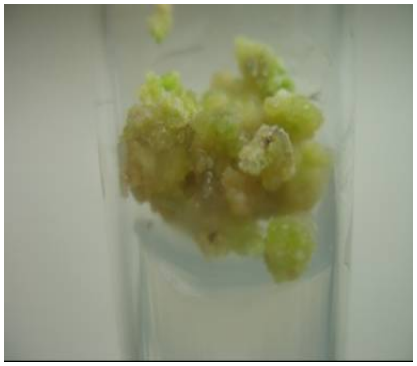


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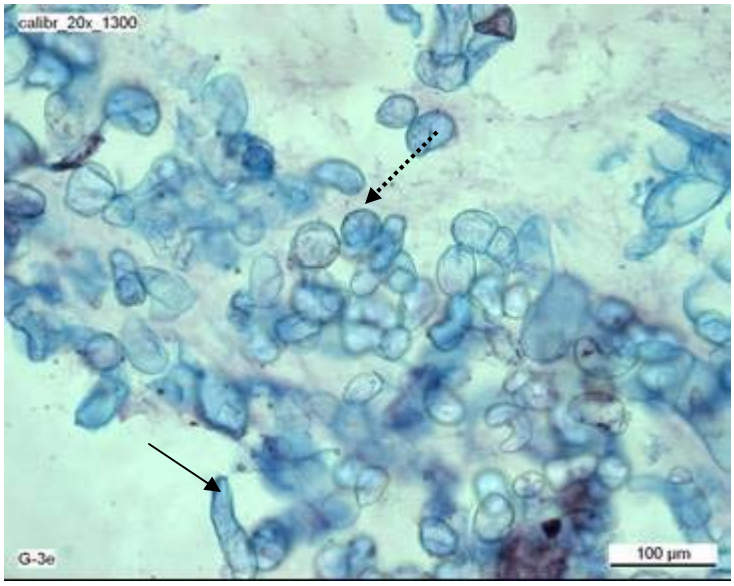
Figure 1.



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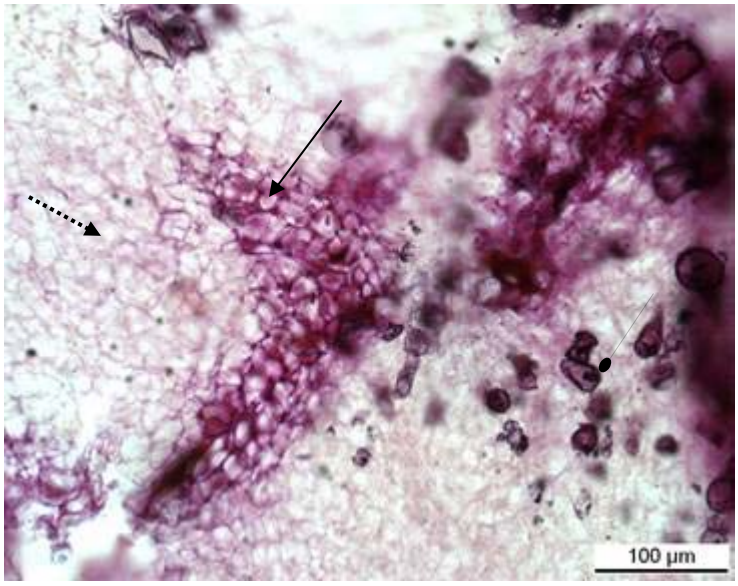

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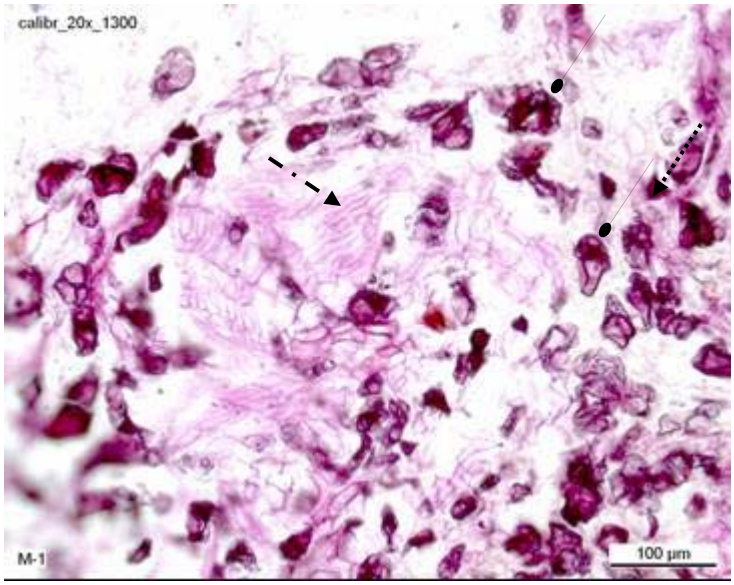

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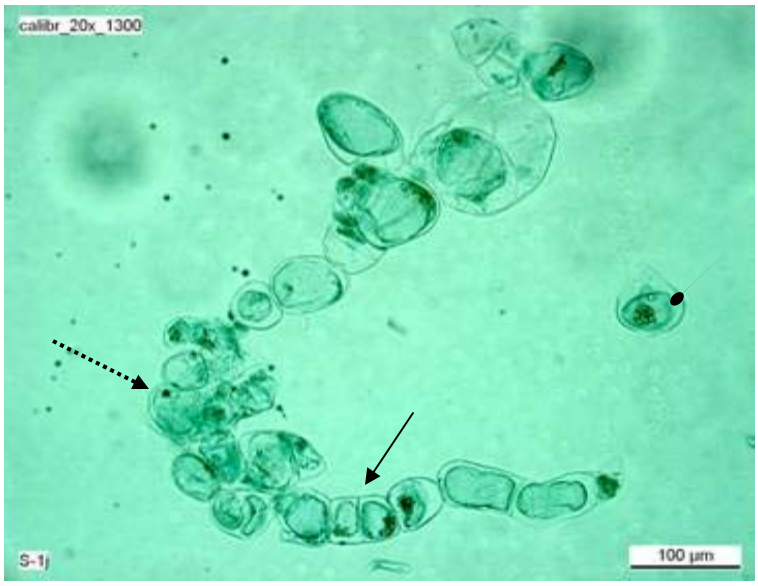
Figure 3.



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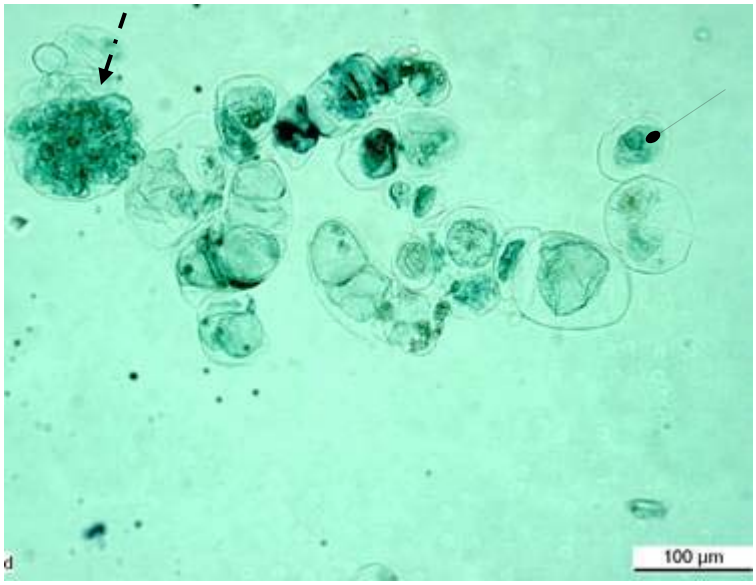

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Figure 4.